

Edaphic and host species influences on *Tuber aestivum* mycelial dynamics in oak forests of the Istrian peninsula, Croatia

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Abstract

Understanding the ecological drivers of *Tuber aestivum* productivity in natural oak forests remains limited despite the species' broad ecological niche and rising economic value. We characterised 65 productive plots of *T. aestivum* associated with *Quercus pubescens* and *Q. cerris* in forest stands of the Istrian peninsula, Croatia. At each plot, we applied a paired sampling design consisting of two systematically collected soil samples: one taken beneath the harvested ascocarp and the other taken 5 m outside the visible *brûlé* or tree crown area, yielding a total of 130 samples. We quantified soil physical–chemical properties (pH and texture), macronutrients (C, Ca, K, N and P), and mycelial abundance by qPCR (ITS2 copies g⁻¹ soil). Across the study area, a positive relationship between clay content and mycelial abundance emerged as the most consistent, highlighting the importance of soil water retention in *T. aestivum* mycelial development. In contrast, higher N levels and imbalanced K: Ca ratios were associated with lower mycelial abundance, particularly in *Q. pubescens* stands, whereas P showed a marginal, host-dependent positive trend detectable only in *Q. pubescens*. Trends of increasing and decreasing median pH and nutrient values were detected between paired samples, with these patterns differing between host tree species. Altogether, our results indicate that *T. aestivum* mycelial development is promoted by clay-rich soils in oak forests and demonstrate the value of paired sampling for disentangling host- and soil-driven processes in wild *T. aestivum* ecosystems.

1. Introduction

The popularity of truffles as an exclusive ingredient in high cuisine and as a valuable trade commodity on global markets has increased over the past few decades (Gryndler et al. 2011; Moser et al., 2017). In parallel, knowledge of their ecological requirements, occurrence and distribution in natural productive areas is being continuously gathered (Zjalic et al., 2020; Daskalopoulos et al., 2025). Truffles are the fruiting bodies of hypogeous fungi belonging to the *Tuber* P. Micheli ex F.H. Wigg. (1780) genus that form ectomycorrhizal (EcM) associations with roots of their host plants (Trappe and Claridge, 2010). Due to numerous synonyms and misidentification, enumerating worldwide species diversity for *Tuber* has been difficult, but it is currently estimated that at least 180 to 230 species belong to this genus (Bonito et al., 2010), of which 13 have commercial value (Reyna and Garcia-Barreda, 2014). The most valuable species among them are considered to be *Tuber magnatum* Picco, *T. melanosporum* Vittad. and *T. aestivum* Vittad. (Reyna and Garcia-Barreda, 2014; Molinier et al., 2016; Bach et al., 2021). The global trade value of these three species accounts for several hundred million USD on the global market, and their wild production has been declining throughout the 20th century (Samils et al., 2008; Riccioni et al., 2016). However, the latter should be taken with caution regarding *T. aestivum*, due to the lack of reliable historical data on production quantities (Büntgen et al., 2011; Splivallo et al., 2012; Stobbe et al., 2013). The market price of summer truffles (*T. aestivum*) when compared to the price of the other two truffle species is lower, but since it can reach 600 € kg⁻¹ on certain markets, they are still gaining in popularity for wild harvesting and cultivation (Molinier et al., 2016; Steidinger et al., 2022) and can play an

economically important role in fostering new livelihood opportunities in the Mediterranean area (Brenko et al., 2022).

T. aestivum is the most widespread truffle species in Europe, encompassing productive areas from southern Sweden to northern Africa, and from Ireland and Portugal to Azerbaijan and the Caucasian region, with the centre of production in temperate forests of central Europe (Molinier et al., 2016; Todesco et al., 2019). It thrives across soils with diverse physical and chemical properties (Chevalier, 2012; Robin et al., 2016) in symbiosis with numerous vascular plant species (Chevalier, 2012; Zambonelli et al., 2015; Santos-Silva and Brigido, 2024). Summer truffles occur from sea level up to 1600 m asl, in neutral or slightly alkaline soils (pH 7–8), but with a reported minimal pH value of 5.9 (Stobbe et al., 2013; Molinier et al., 2016; Hilszczańska et al., 2019). Soil texture in forest productive areas is variable, ranging from silty-clay to clayey-silt, with clay content up to 52.8%, depending on the origin of bedrock (Chevalier and Sourzat, 2012).

Despite the existing knowledge and success in cultivation of commercial black truffles, i.e., *T. melanosporum* and *T. aestivum* (Riccioni et al., 2016), and promising results in cultivation of *T. magnatum* (Bach et al., 2021; Čejka et al., 2023), shifts in climatic conditions make these efforts challenging (Le Tacon, 2016; Čejka et al., 2020). The negative impact of high temperatures and drought during the summer months in the centre of *T. aestivum* climatic range, where such conditions reduce its wild production and may even stop it under warming scenarios of 2–3°C, was observed (Steidinger et al., 2022). Likewise, based on models predicting hotter and drier summers, the production of *T. melanosporum* in forest ecosystems is expected to continue declining, becoming restricted to the centre of its ecological range (Reyna-Domenech and Garcia-Barreda, 2009; Baragatti et al., 2019; Oliach et al., 2020).

Successful fructification of *T. aestivum* depends, except for climate (Steidinger et al., 2022), on edaphic conditions (Le Tacon, 2016; Bragato et al., 2021; Wilgan et al., 2025) and the presence of appropriate host species (Santos-Silva and Brígido, 2024). Soil nutrients influence soil pH and stimulate mycelial growth (Hilszczańska et al., 2021; García-Montero et al., 2024) and may differentiate *T. aestivum* productive areas from those belonging to *T. melanosporum* (Chevalier and Sourzat, 2012). The ability of EcM fungi to modify their habitat (niche construction) by altering soil pH, carbon (C), calcium (Ca), potassium (K) (García-Montero et al., 2009 and 2024), nitrogen (N) and phosphorus (P) (Nehls and Plassard, 2018; Lin et al., 2024) to productive zones surrounding the host root system has been observed.

Among various host species that form mycorrhiza with *T. aestivum* (Santos-Silva and Brigido, 2024), pubescent oak (*Quercus pubescens* Willd.) and Turkey oak (*Quercus cerris* L.) are considered to be economically important, since they can be used in the establishment of truffle orchards (Bordács et al., 2019; Simeone et al., 2019; Čejka et al., 2020). Pubescent oak is native to Europe and Asia Minor, grows from sea level up to 1500 m asl, on poor and eroded forest soils, mostly on sunny slopes, can tolerate winter cold up to a certain point, and it requires high summer temperature with little rain (Bordács et al.,

2019). Turkey oak is a widely distributed species in south-east Europe and Asia Minor that shows moderate resistance to drought (Simeone et al., 2019). Turkey oak prefers basic soils on higher altitudes (from lowlands up to 2200 m a.s.l.) and does not tolerate maritime exposures, although the summer droughts in Mediterranean evergreen forests are not the limiting factor for its occurrence (Simeone et al., 2019). Wide genetic variability of pubescent and Turkey oak could respond to environmental stress induced by climate change and preserve their natural distribution (Lados et al., 2024), which could be significant for truffle cultivation when they're used as a host species for *T. aestivum* mycorrhiza.

Wider knowledge on geographical, climate and edaphic conditions that influence the growth of *T. aestivum* and their tree hosts may facilitate the development of suitability maps of the natural occurrence of truffles. Such maps could be used both to monitor wild production and to promote cultivation (Moser et al., 2017) by identifying optimal sites for spontaneous occurrence and guiding habitat conservation and management (Serrano-Notivoli et al., 2016). Although extensive data exist on the dependence of truffle fruiting body production on climatic conditions in orchards, those dependencies in natural forests remain insufficiently understood (Le Tacon, 2016; Steidinger et al., 2022). General predictive maps of *T. aestivum* natural occurrence in Europe have been developed by Molinier et al. (2016), Riccioni et al. (2019) and Santos-Silva and Brígido (2024), with the maps of Čejka et al. (2020) for the Czech Republic being the most advanced. Based on our knowledge, and despite the general assumption that *T. aestivum* is a species with the widest ecological niche, such maps have not been developed for Croatia, possibly due to the under-explored ecology of *T. aestivum* in some of the central parts of the Mediterranean (Zjalić et al., 2020; Daskalopoulos et al., 2025). This contrasts with the economic and cultural importance truffles have in parts of Croatia that have a long tradition in truffle exploitation, such as the Istrian peninsula (Zjalić et al., 2020), where truffle productive oak forests are described as pubescent oak and oriental hornbeam (*Carpinus orientalis* Mill.) forests (*Quercus-Carpinetum orientalis* H-ić 1939) (Vukelić and Rauš, 1998) and where *Q. pubescens* is described as an edificatory species with a wide area of occurrence that overlaps with *Q. cerris* (Supplement Fig. 1).

In order to overcome these knowledge gaps, the main objective of this study was to assess the influence of geographical and edaphic conditions on the mycelial development of *T. aestivum* in the underexplored, naturally productive oak forests of the Istrian peninsula, Croatia. Specifically, we tested the influence of altitude, soil alkalinity, soil texture and macronutrient availability (C, Ca, N, K, P) on mycelial development of *T. aestivum*, and evaluated whether these relationships differ between *Q. pubescens* and *Q. cerris*. Using paired soil samples collected beneath harvested ascocarps (IN) and from adjacent non-productive zones (OUT), we tested whether soil properties differed consistently within plots, helping to disentangle host-associated effects from background soil variations in wild truffle ecosystems.

2. Materials and methods

2.1. Research area

The research area consisted of natural productive areas of *Tuber aestivum* in the Istrian peninsula, north of the Adriatic (45°14'12.60" N 13°56'11.39" E), where previous potential sampling locations were identified according to Forest Management Plans of Croatian Forests Ltd. (2017) and *T. aestivum* known ecosystem conditions. The study area was limited by the administrative borders of Istrian County, Croatia (Fig. 1). More precisely, it borders the Republic of Slovenia to the north, Primorje-Gorski Kotar County to the north-east and is surrounded by the Adriatic to the east, south and west, encompassing a total area of 2820 km² (Istrian County, 2020). The altitude of sampling locations ranged between 7 and 443 m asl, from which *Q. pubescens* sampling plots ranged between 7 and 421 m asl with a median of 282.5 m asl, and from 284 to 443 m asl with a median value of 376 m asl for *Q. cerris* sampling plots (Supplement Table 1).

The climate of the peninsula is characterised as the temperate Mediterranean climate with warm and hot summers (Istramet, 2019), with mean temperatures varying from 3°C in winter months to 22°C in summer months, although the historical absolute values were – 18°C and 39°C (Croatian Meteorological and Hydrological Service, 2025b). Average yearly temperature (Supplement Table 2) recorded from weather station Poreč (WS1) of the sampling year was 15.5°C, while 13.1°C was recorded from weather station Pazin (WS2) (Croatian Meteorological and Hydrological Service, 2025a). The average monthly precipitation in the same year recorded from WS1 was 87.5 mm, while from WS2 was 104.2 mm (Croatian Meteorological and Hydrological Service, 2024b). On both weather stations, most rainfall was recorded during the summer and autumn months. The average soil temperature at a 10 cm depth recorded during the sampling period (June, July, August and September 2023) was 25.5°C for WS1 and 24.2°C for WS2 (Croatian Meteorological and Hydrological Service, 2024a and Supplement Fig. 2).

2.2. Plot selection and soil sampling procedure

Field research was conducted from mid-June to the end of September 2023. Following the existing knowledge on natural habitat conditions and symbiont trees of *T. aestivum*, and with the help of forest management plans, potential productive locations were identified, spreading evenly throughout the research area. Sampling plots were more precisely defined by the local truffle hunters, for which exact locations will not be provided following their requests. The potential sampling plots were scanned objectively without any preference to host tree species. Within the area of 908 km², a total of 65 plots were identified as productive, out of which 37 plots belonged to *Q. cerris* as host tree species and 28 to *Q. pubescens*. No *T. aestivum* ascocarps were found in symbiosis with *Quercus ilex* L. and *Pinus halepensis* Miller during the sampling period, although local truffle hunters reported the productivity of those hosts. Productive plots had a circular shape, with a 5 metre radius from the found *T. aestivum* ascocarp. In order to avoid ambiguity, the same two trained truffle dogs were used for the majority of sampling (55 plots), while 10 plots were identified by the other three dogs. Soil sampling was done using a traditional metal truffle shovel used in the area. From each sampling plot paired soil samples were taken. One half of the pair was sampled from the zone beneath the found ascocarp (IN) and the other half from the zone 5 m apart from the visible *brûlé* or tree crown (OUT), at a depth of 5–15 cm (discarding the top humus layer, stones and other plant material), resulting in a total of 130 samples. The

minimum distance between sampling plots was 30 m. In case multiple ascocarps were found within a smaller distance between each other or between host trees, composite soil samples were taken (Fig. 2). Between the sampling zones, sampling tools were sterilised by using commercial sanitiser in order to avoid cross-contamination of samples. Each soil sample contained approximately 1 litre of soil stored in a plastic bag. For each sampling location, the following data were recorded: date, location, altitude, host tree species, number of ascocarps found and average diameter of ascocarp. Every day after the field work, each soil sample was separated into two 0.5 litre samples, one of which was frozen to -18°C the same day, while the other was left for drying before physical-chemical analysis.

2.3. Soil physical-chemical analysis

The pH of the analysed samples was determined using a 1:5 soil weight/water volume ratio (HRN ISO 10390:2005). C content was detected by burning 50 mg of grounded sample in the Solid Sample Combustion Unit (SSM-5000A) on TOC-L analyzer (Shimadzu Corporation, Kyoto, Japan). N content was determined by the Kjeldahl method (Kjedahl, 1883). For digestion, 1 g of grounded sample, 12 mL of H₂SO₄, and 2 KJTabs™ tablets were used and a 1 h digestion at 420°C program was performed. After digestion and cooling, 30 mL of H₃BO₄ and 50 mL of NaOH were used for distillation on UDK 149 Nitrogen Analyzer (VELP Scientifica Srl., Usmate Velate, Italy). Titration was performed using 0,1 N HCl. The analysis of Ca, K and P was performed by ICP-OES (Shimadzu Corporation, Kyoto, Japan) after microwave digestion (Ethos UP, Milestone Srl, Milan, Italy). Microwave digestion of the samples was performed using 6 mL of HNO₃ and 2 mL of H₂O₂ added to 250 mg of the sample. The program was set for a 25 min ramp to 200°C and held for 15 min. Microwave digestion was performed in two steps. In the first step, 6 mL of HNO₃, 2 mL of H₂O₂, and 0.4 mL of HF were added to the 200 mg of grounded sample. The program was set for 15 min temperature ramp up to 190°C, holding that temperature for 20 min. After cooling, in the second step, 5 mL of 4% H₃BO₃ were added. Temperature ramp lasted eight minutes up to 160°C and was held for seven minutes. After completed digestion, samples were left to cool at room temperature and quantitatively transferred and diluted to 25 mL with ultrapure water. The detection of elements was performed using an ICP-OES with both axial and radial viewing (ICPE-9800 Shimadzu, Kyoto, Japan). Operating parameters were as follows: 1.15 kW of a RF power, 12 L/min of plasma flow rate, 0.5 L/min of auxiliary gas flow rate and 0.5 L/min of nebuliser flow rate. Sample solutions were introduced into the plasma using a concentric nebuliser and a cyclonic type spray chamber. Argon (99.999% pure, Linde Gases, Ananindeua, PA, Brazil) was used to purge the optics and to form the plasma.

Since the mechanical analysis of the soil was done considering a silt - sand limit of 63 μmeters, the German 'Standortserkundungsanweisung' (SEA 1974) soil texture classification for forest soils was used to describe the soil types of the sampled zones following the plotting instructions given by the Moeys (2024).

2.4. DNA extraction and PCR

Frozen portions of samples were delivered to the laboratory for further molecular analysis. They were freeze-dried for ~ 48 h, sieved through a 2 mm mesh and homogenised by grinding to fine powder. Total soil genomic DNA was extracted from processed soil samples using a NucleoSpin® soil DNA isolation kit (Macherey–Nagel, Düren, Germany) following the manufacturer's instructions. In total, 500 mg of soil was extracted from each homogenised sample. Ascocarp DNA was extracted using 20 mg of dry weight of each ascocarp with the same soil DNA isolation kit (Macherey–Nagel, Düren, Germany). All the ascocarps were confirmed to be *Tuber aestivum* by PCR with specific primers TaO F (5'-CCCCGCCGAAATGTATGG-3') following Gryndler et al. (2013), and ITS4LNG (5'-TGATATGCTTAAGTTCAGCGGG-3') following the approach of Paolocci et al. (1999). The final concentrations in 25 µl PCR reactions were: 50 ng template, 200 µM of each nucleotide, 2.75 mM MgCl₂, primers at 250 nM, 0.025 U µL⁻¹ polymerase (DreamTaq Green, Thermo Scientific, Waltham, MA, USA) in 1× buffer. PCR cycling conditions were: 5 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 60°C, 30 s at 72°C, and a final extension step at 72°C for 7 min.

For the quantification of *T. aestivum* mycelium in the soil samples, we used primers and probe developed by Gryndler et al. (2013) to amplify the ITS fragment of 73 bp. Prior to qPCR, DNA extracts were diluted 10 times to avoid PCR inhibition. All the samples were processed in technical triplicates. Negative controls for DNA extractions and PCRs were included in each plate. The reaction contained 5 µl of template, 10 µl of 2× iTaq™ Universal Probes Supermix (Bio-Rad®), 500 nM of each oligo, 200 nM of the hydrolysis probe and ultrapure water in a final reaction of 20 µl volume. The cycling conditions in a QuantStudio 1 Real-Time PCR System (Applied Biosystems) were: initial denaturation for 30 sec at 95°C, followed by 40 cycles of 5 sec denaturation at 95°C and 34 sec of annealing and synthesis at 60°C.

For the standard curve, we used a pGEM-T plasmid containing one copy of the same fragment of the ITS gene amplified in the samples (73 bp). Serial dilutions of the plasmid with a known number of molecules were performed. Each plate included a standard curve with six points of tenfold dilutions, with the most concentrated of 2.403×10^6 ITS2 copies. The lowest dilution of the plasmid mixture for the standard curve corresponded theoretically to 24 copies of ITS2. Samples with Ct values that were above the Ct values of the lowest plasmid dilution were not considered for the real signal. This selection was done for each plate according to its standard curve. Absolute soil mycelium quantification was estimated by interpolating Ct values on the corresponding standard curve and expressed as ITS copies/g of soil.

2.5. Statistical analysis

The relationships between altitude, soil alkalinity, soil texture and nutrient availability (explanatory variables) were first assessed in relation to the mycelial quantity and host tree species. Analyses were initially conducted across the entire study area, followed by analyses stratified by host tree species and IN and OUT sampling zones. To assess overall multivariate relationships between sets of variables, a permutational multivariate analysis of variance (PERMANOVA) was performed using the Community Ecology Package (*vegan* library) v.2.6-8 and its dependencies (Oksanen et al., 2022). Data standardisation was performed using the *decostand* function on a dataframe that contained explanatory

variables (altitude, alkalinity, sand, clay, C, Ca, N, P and K). Homogeneity of data was confirmed by calculating dispersion. To further explore and quantify the relationships between individual explanatory variables and soil mycelial quantity and host tree species, linear regression analysis was conducted. These models were also used to assess differences associated with host tree species. During linear regression, a square root transformation was done to handle the skewed data of mycelial quantity. While PERMANOVA allowed the assessment of multivariate structure, linear models enabled the identification of specific drivers of mycelial quantity. Graphs were generated using the *ggplot2* package v.4.0.2 (Wickham, 2016). Statistical analysis was done in RStudio.

3. Results

3.1. Whole research area analysis

Across the whole research area, PERMANOVA results indicate that soil texture was significantly associated with mycelial quantity ($p = 0.012$, $R^2 = 0.03$), while soil nutrient composition influenced host tree species distribution ($p = 0.001$, $R^2 = 0.04$). Linear regression analysis, performed to display individual nutrient effects (Table 1), suggests a significant positive correlation between mycelial quantity and clay content ($p = 0.004$), whereas no significant relationships were observed between mycelial quantity and other explanatory variables. In contrast, host tree species distribution showed a significant correlation with altitude, P, K, C, N and Ca (for all $p < 0.001$).

Table 1

Results of linear regression models conducted on the whole research area, for both host tree species' productive (IN) and non-productive (OUT) sampling zones. The table displays only significant results of the performed models (Sign. codes: '***' $p < 0.001$, '**' $p < 0.01$, '*' $p < 0.05$, '.' $p < 0.1$)

	Estimate	Std. Error	t value	Pr (> t)
Model 1 (mycelial quantity ~ soil pH, texture and nutrients). Model $p = 0.028$, Adjusted $R^2 = 0.07$				
(Intercept)	240.12	2372.58	0.10	0.919
clay	32.23	11.14	2.89	0.004 **
Model 2 (host tree species ~ altitude). Model $p < 0.001$, Adjusted $R^2 = 0.27$				
(Intercept)	0.97	0.09	10.71	< 0.001 ***
altitude	0.00	0.00	7.10	< 0.001 ***
Model 3 (host tree species ~ soil pH, texture and nutrients). Model $p < 0.001$, Adjusted $R^2 = 0.41$				
(Intercept)	1.07	0.87	1.22	0.223
C	0.02	0.00	5.17	< 0.001 ***
Ca	3.79	0.92	4.10	< 0.001 ***
N	-0.42	0.07	-5.37	< 0.001 ***
K	0.09	0.01	6.00	< 0.001 ***
P	0.41	0.06	5.89	< 0.001 ***

3.2. Host-specific analysis

While comparing IN zones of both host tree species, and OUT zones, separately, no significant differences were found in mycelial quantity, although lower amounts of mycelial quantity within *Q. pubescens* sampling zones were observed when compared to *Q. cerris* sampling zones. Median amount of ITS2 copies g^{-1} soil (Fig. 3) in *Q. pubescens* IN zones was 3.03×10^6 , while in *Q. cerris* that value was 3.77×10^6 . In OUT zones, the median amount of ITS2 copies g^{-1} soil was 2.20×10^4 and 8.34×10^4 , *Q. pubescens* and *Q. cerris*, respectively. Significant differences were confirmed within sampling zones and host tree species, respectively, and they both resulted in $p < 0.001$.

Soil pH (Supplement Table 1) values in the IN zones of *Q. pubescens* ranged from 6.69 to 7.46, while for *Q. cerris* IN zones, those values ranged from 6.57 to 7.83. For OUT zones, pH values ranged from 6.00 to 7.56 and 5.99 to 8.05, *Q. pubescens* and *Q. cerris*, respectively. The differences in pH medians between host tree species and sampling zones resulted in no significance.

Soil texture triangles (Fig. 4) indicated relatively homogeneous soil textures across the study area, varying from loamy or silty clay dominated soils, with most productive plots occurring in silt / silty loam soils.

3.3. Sampling zone-specific analysis

Linear regression models (Table 2) provided more detailed results after comparing the edaphic properties between host tree species sampling areas and sampling zones (IN and OUT). Within *Q. pubescens* IN zones, mycelial quantity showed a significantly positive correlation with Ca content ($p < 0.001$), and a significant negative correlation with pH ($p = 0.026$, Fig. 5). Marginal positive and negative correlations were also observed between mycelial quantity and P ($p = 0.083$) and N content ($p = 0.087$), respectively. In *Q. pubescens* OUT zone, mycelial quantity was positively correlated with clay content ($p = 0.009$) and negatively correlated with K content ($p = 0.047$).

Within *Q. cerris* IN zones, mycelial quantity was positively correlated with clay content ($p = 0.006$), while no other significant relationships were found in either IN or OUT zones. Here, comparisons of differences in observed parameters between sampling zones (IN and OUT) were also tested, stratified by host tree species, revealing no significant differences.

Table 2

Results of the linear regression model conducted by separating *Q. pubescens* and *Q. cerris* productive plots and sampling zones. **Model 1** - *Q. pubescens* productive (IN) zones. **Model 2** - *Q. pubescens* non-productive (OUT) zones. **Model 3** - *Q. cerris* productive (IN) zones. The table displays only significant results of the performed linear regression models (Sign. codes: '***' $p < 0.001$, '**' $p < 0.01$, '*' $p < 0.05$, '.' $p < 0.1$)

	Estimate	Std. Error	t value	Pr (> t)
Model 1: <i>Q. pubescens</i> IN zones (mycelial quantity ~ soil pH, texture and nutrients)				
Model p = 0.039, Adjusted R² = 0.32				
(Intercept)	22826.59	7725.32	2.95	0.008 **
pH	-2684.11	1115.64	-2.40	0.026 *
Ca	33498.39	7739.10	4.32	< 0.001 ***
N	-1052.51	583.91	-1.80	0.087 .
P	2298.53	1254.67	1.83	0.083 .
Model 2: <i>Q. pubescens</i> OUT zones (mycelial quantity ~ soil pH, texture and nutrients)				
Model p = 0.018, Adjusted R² = 0.39				
(Intercept)	5085.84	3033.10	1.67	0.110
clay	45.47	15.85	2.86	0.009 **
K	-123.10	58.04	-2.12	0.047 *
Model 3: <i>Q. cerris</i> IN zones (mycelial quantity ~ soil pH, texture and nutrients)				
Model p = 0.026, Adjusted R² = 0.26				
(Intercept)	6210.89	5438.57	1.14	0.263
clay	52.06	17.82	2.92	0.006 **

Lastly, differences in pH, soil texture and nutrient amounts were observed between host tree species and sampling zones, respectively (Table 3), excluding observations towards mycelial quantity. Linear regression models display significant differences in IN zones for K ($p = 0.004$), Ca ($p = 0.015$) and P ($p = 0.077$), while in OUT zones differences were observed only between K ($p = 0.001$) and P ($p = 0.071$, Fig. 6).

Table 3

Results of linear regression models conducted to compare differences between all productive (IN) and all non-productive (OUT) zones grouped by host tree species. The table displays only significant results of the performed linear regression models (Sign. codes: '***' $p < 0.001$, '**' $p < 0.01$, '*' $p < 0.05$, '.' $p < 0.1$)

	Estimate	Std. Error	t value	Pr (> t)
Model 1 (Ca ~ IN zones). Model p = 0.015, Adjusted R² = 0.07				
(Intercept)	0.03	0.00	3.71	< 0.001 ***
Ca	0.02	0.01	2.49	0.015 *
Model 2 (P ~ IN zones). Model p = 0.077, Adjusted R² = 0.03				
(Intercept)	0.16	0.09	1.71	0.090 .
P	0.23	0.12	1.79	0.077 .
Model 3 (K ~ IN zones). Model p = 0.004, Adjusted R² = 0.10				
(Intercept)	12.17	0.41	29.44	< 0.001 ***
K	1.62	0.54	2.96	0.004 **
Model 4 (P ~ OUT zones). Model p = 0.071, Adjusted R² = 0.03				
(Intercept)	0.16	0.09	1.69	0.095 .
P	0.23	0.12	1.83	0.071 .
Model 3 (K ~ OUT zones). Model p = 0.001, Adjusted R² = 0.14				
(Intercept)	11.71	0.44	26.50	< 0.001 ***
K	2.01	0.58	3.43	0.001 ***

4. Discussion

Within this study, our results reveal that the influence of soil alkalinity, texture and nutrients on mycelial quantity differs between *Q. pubescens* and *Q. cerris* productive zones, while altitude has a direct influence only on host tree species composition, rather than mycelial quantity directly. Also, significant differences in nutrient composition were observed between host tree species and sampling zones, respectively.

Our results indicate that the mycelial quantity of *T. aestivum* in general is primarily influenced by clay content. Clay particles exhibit strong water retention capacity and, as reported in previous research by Querejeta (2016), Boeraeve et al. (2018) and by Hagenbo et al. (2020), soil moisture has a positive effect on EcM mycelia abundance. The significant positive relationship between mycelial quantity and clay

content across the entire study area ($p = 0.004$) and within *Q. cerris* IN zones ($p = 0.006$) and *Q. pubescens* OUT zones ($p = 0.009$) suggests that clay content may promote mycelial biomass accumulation. This finding is consistent with a study conducted in *T. melanosporum* orchards where clay content was shown to be a stronger predictor of truffle production under low irrigation scenarios (Ponce et al., 2014). Having in mind that *T. melanosporum* and *T. aestivum* have opposite production seasons (winter vs. summer, respectively), clays' water retention capacity could be even more emphasised in water-distressed natural forest stands during summer months. The positive role of water availability on *T. aestivum* mycelial quantity was also observed by Piñuela et al. (2021) in irrigation experiments.

Although altitude did not have a direct impact on mycelial quantity, it significantly influenced host tree species distribution ($p < 0.001$, median altitudes of sampling locations 282.5 m asl and 376 m asl, *Q. pubescens* and *Q. cerris*, respectively). We assume that higher precipitation and lower average air and soil temperatures observed from the weather station connected with *Q. cerris* sampling locations (Supplement Table 2 and Supplement Fig. 2) could promote previously discussed clay's moisture retention capacity. These findings are consistent with previously reported ecological preferences of *Q. pubescens* and *Q. cerris* regarding the site preferability towards altitude, temperature and soil moisture (Bordács et al., 2019; Simeone et al., 2019) and confirm the host tree species' distinct habitat association within the study area.

In addition to altitude, host tree species distribution is influenced by soil nutrient composition. The observed differences in nutrient levels among host tree species (Fig. 6) are likely related to the different bedrock types formed during Jurassic-Cretaceous-Palaeogene periods, which influenced the genesis of different soil types across the Istrian peninsula (Peh et al., 2003). Furthermore, the differences in nutrients reported by Peh et al. (2003), which are a result of soil genesis, are also confirmed within this study: elevated pH, Ca and K amounts in central parts of the peninsula (all of *Q. cerris* sampling zones) and higher P amounts in western parts (most of *Q. pubescens* sampling zones).

No significant differences were observed in soil alkalinity, texture and nutrient contents between sampling zones and host tree species, respectively. However, light trends in median amounts of pH and nutrient contents within the sampling zones can be observed. In contrast, mycelial quantity significantly differed between the sampling zones and relatively large amounts of mycelial quantity were observed within some of the OUT zones (Fig. 3). This pattern can be explained by the sampling design. OUT zones were man-made decisions based on visual observations of productive plots' surroundings. Although no *T. aestivum* ascocarps were found inside the OUT zones, the danger of soil sampling towards the direction of future productive location or mycelial abundance existed, which probably would not be the case in truffle orchards. Also, since the distance between the IN and OUT sampling zones was relatively low (> 5 m), more coherent alkalinity levels and nutrient contents were expected.

Within *Q. pubescens* productive areas, trends in a slight increase of pH, Ca, K, N and P median levels between the IN and OUT sampling zones were observed. Within *Q. cerris* productive areas, only a slight increase in Ca and N and a slight decrease in P median amounts were observed between these zones.

Since previous studies (García-Montero et al., 2009 and 2024; Nehls and Plassard, 2018; Lin et al., 2024) have suggested that truffles may modify their surrounding soil environment through niche construction processes, our results don't provide clear evidence supporting this mechanism, due to the lack of significant differences in alkalinity and nutrient amounts within sampling zones of each host tree species. Nevertheless, the observed trends and the way they differ between the host tree species deserve mentioning and special attention in future studies.

The negative estimated effect of N within our study suggests a potential sensitivity of *Q. pubescens* systems to elevated N availability. N is an essential macronutrient in plants, and its availability can trigger the root water uptake (Ding et al., 2018; Araus et al., 2020). In scenarios of water limitation and high N availability, Fusaro et al. (2017) observed species-specific stress response between deciduous (*Fraxinus ornus* L.) and evergreen (*Q. ilex*) species in the Mediterranean region, but both benefited from stomatal conductivity triggered by available N amounts. In our study, although no significant differences in N content were detected between the host tree species (with a slight increase in its median value within *Q. pubescens* sampling zones), N's significance was observed in pubescent oak productive areas with less average rainfall and higher air and soil temperature. It seems that N may play a beneficial role for the host trees in water deficit scenarios. In contrast, a marginal negative correlation was observed between mycelial quantity and N within *Q. pubescens* IN zones ($p = 0.087$). Decreased C supply from plants towards EcM fungi was previously observed with high N availability in coniferous boreal and nemoral forests of Europe by Högborg et al. (2021) and Jørgensen et al. (2024). Also, in a research conducted in the *Q. pubescens* forest stand in Northern Italy by Innangi et al. (2020), lower total N amounts were observed inside *brûlés* (compared to adjacent outer parts), insinuating *T. aestivum* nitrophobic properties. EcM fungi are dependent on photosynthesis-derived carbohydrates (Nehls et al., 2010; Smith et al., 2017; Deng et al., 2023) and the lack of C flow towards fungal mycelia, as a consequence of higher N availability in soil, could explain the observed marginal negative correlation. Since N can be beneficial for the mycelia development of EcM fungi up to a certain threshold (Jørgensen et al., 2024), our results indicate that in the Istrian peninsula, this threshold lies near the median amount of N within *Q. cerris* IN zones ($4.48 \text{ g kg}^{-1} \text{ soil}$), where no correlations between mycelial quantity and N were observed.

Within *Q. pubescens* IN zones, mycelial quantity showed a positive correlation towards Ca and negative towards pH (Fig. 5). Ca is considered to be one of the most important elements for the development of truffles since carbonate (i.e. CaCO_3 and Ca^{2+}) availability in soil regulates soil pH and supports the development of truffle mycelia (Hilszczańska et al., 2019). Gryndler et al. (2017), in their research conducted in a deciduous mixed forest with European hornbeam (*Carpinus betulus* L.), reported the positive correlation between CaCO_3 in soil and *T. aestivum* mycelial quantity. In addition, the same observations were repeated by García-Montero et al. (2024) on multiple locations in Spain where *T. aestivum* produced fruiting bodies in symbiosis with *Quercus* spp. Furthermore, Habtemariam et al. (2024) observed in the research conducted in Hungary in *T. aestivum* orchard with *Carpinus betulus*, *Corylus avellana* L., *Pinus nigra* Arnold, *Quercus petraea* L., *Q. robur* L. and *Q. pubescens*, that soil pH regulation by calcium-carbonates also proves to be efficient in controlling the undesired root

colonisation by competitive mycorrhizal fungi that thrive in slightly acidic environments. *T. aestivum* mycelia in symbiosis with *Q. pubescens* (within our research area) develop in basic soils with significantly less soil Ca amount compared to *Q. cerris* productive areas (Table 3 and Fig. 6), which, as a result, had less observed mycelial quantity within *Q. pubescens* IN zones. Positive correlation of mycelial quantity towards Ca and negative towards pH confirms the high demand towards Ca, which is best exhibited in slightly lower soil pH values.

The influence of K was only observed in *Q. pubescens* OUT zones ($p = 0.047$, with negative direction). In plants, K and Ca display a synergistic relationship. While K controls photosynthetic assimilation, nutrient uptake and turgor (Sardans and Peñuelas, 2021), Ca strengthens cell walls and membranes (White and Broadley, 2003) needed for effective nutrient flow and internal hydrostatic pressure (Lew, 2011). In litter, K displays high mobility properties (Hu et al., 2021; Spohn and Berg, 2023; Moran-Rodas et al., 2025), and it is the most abundant macronutrient in soils, but the majority of its content is bound in minerals and rocks, making it less than 2% available for plant uptake in the form of solution or exchangeable phase (Sparks, 2001). The EcM fungi's property of mobilising K from clay minerals has also been observed (Sardans et al., 2023). In our study, except for C, K is found in the highest quantities among other researched nutrients in all of our soil samples. In soils, K and Ca display antagonistic properties during the absorption process; high levels of K can displace Ca from exchange sites in the soil or inhibit its uptake into fungal cells (Rhodes et al., 2018), which directly weakens mycelial development. The K: Ca ratio in *Q. pubescens* OUT zones was 2.2 times higher than in *Q. cerris* OUT zones (950:1 vs. 420:1, respectively). Following our discussion of the essential role of Ca on *T. aestivum* mycelial quantity, the observed negative influence of K on mycelial quantity in *Q. pubescens* OUT zone is indirect, since its elevated levels inhibit Ca uptake in the scenario of lower Ca median. In *Q. pubescens* IN zone, non-significant mycelial quantity correlation towards K was observed, although the K: Ca ratio was slightly lower than in OUT zone (850:1), which suggests that the threshold of negative influence of K on Ca uptake lies closely above this ratio. These ratios should be taken with caution, due to the comparison of total element amounts, not bioavailable amounts.

Positive correlation between P and mycelial quantity was observed within pubescent oak IN zones, where the median P value was relatively higher than in Turkey oak IN zones, displaying mycelial dependency towards this nutrient. Also, significant differences in P amounts were observed between host tree species' productive and non-productive zones, respectively. The research on the specific effect of P towards mycelial development of EcM hypogeous fungi is still scarce nowadays, compared to the research of P influence towards arbuscular mycorrhiza (Han et al., 2022; Yurkov et al., 2025) or other ectomycorrhizal epigeous fungi (Köhler et al., 2018; Almeida et al., 2019; Bashian-Victoroff et al., 2025). In general, P is considered to be a very important macronutrient both for plant and mycorrhizal development, due to its structural role in cell membranes (Khan et al., 2023). In soil, P in its elementary state is not available for plant uptake, highlighting the importance of mycorrhizas' ability to dissolve the elementary P by enzymatic activities into forms available to plant uptake, like phosphates (Plassard and Dell, 2010). Furthermore, it is considered that large amounts of available P in soil weaken the plant dependency toward EcM fungi, which consequently inhibits EcM mycelia development (Almeida et al.,

2023). In P-deficient soils, the EcM fungi's property of scavenging the soil for nutrients has been observed, resulting in increased extraradical mycelia development and higher mycorrhization rate (Hagenbo et al., 2020; Almeida et al, 2023). Following these observations within this study, relatively more mycelia was observed in *Q. cerris* productive zones where a lower median P value was detected.

Within *Q. cerris* sampling plots, the only observed mycelial quantity dependency was towards clay content. Non-observed mycelial dependencies towards Ca, K, N and P in *Q. cerris* productive zones, in contrast to *Q. pubescens* productive zones, suggest that *T. aestivum* in symbiosis with *Q. cerris* develops in soils with well-balanced nutrient composition and texture that promotes water retention, resulting in 18.8% more mycelial quantity (in median value), compared to *Q. pubescens* IN zones. Petrova et al. (2024) had similar conclusions in a study conducted in Western Bulgaria, although their evaluation was based on ascocarp production, not mycelial quantity. In their research, the presence of *T. aestivum* ascocarps in a mixed broad-leaved and conifer forest with predominance of *Quercus* spp., was most abundant in silty clay loam soils of moderate water-retention capacity, favourable nutrient ratios and low P amounts. They also discussed the benefit of high N amounts in their soil samples, but their median N amount was 1.4 times lower than in our study.

5. Conclusions

This research confirms that the abundance of *Tuber aestivum* mycelia in *Quercus pubescens* and *Q. cerris* productive forest sites is influenced by two distinct, host-dependent niche regimes. Mycelial development under *Q. pubescens* is constrained by elevated N and P concentrations, and high K: Ca ratios, whereas *Q. cerris* supports higher mycelial quantities under more balanced nutrient conditions, and higher clay content. These results show that *T. aestivum* responds to specific combinations of soil alkalinity, texture and macronutrient availability rather than to individual edaphic factors in isolation. By clarifying the fine-scale edaphic mechanisms regulating mycelial development across contrasting oak hosts, this study provides a mechanistic basis for interpreting spatial variability in *T. aestivum* performance on soil–host interactions in Mediterranean truffle ecosystems. The observed host-specific trends in increase and decrease in soil pH and macronutrient amounts deserve to be subjected to future research. Also, since the *T. aestivum* population is considered to be co-driven by climate, site-specific meteorological data (i.e. daily average temperature and precipitation) would help better understand the soil-host-fungi interaction.

Statements and Declarations

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Competing Interests

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

Conflict of interest

The authors declare that they have no conflict of interest.

Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Figures

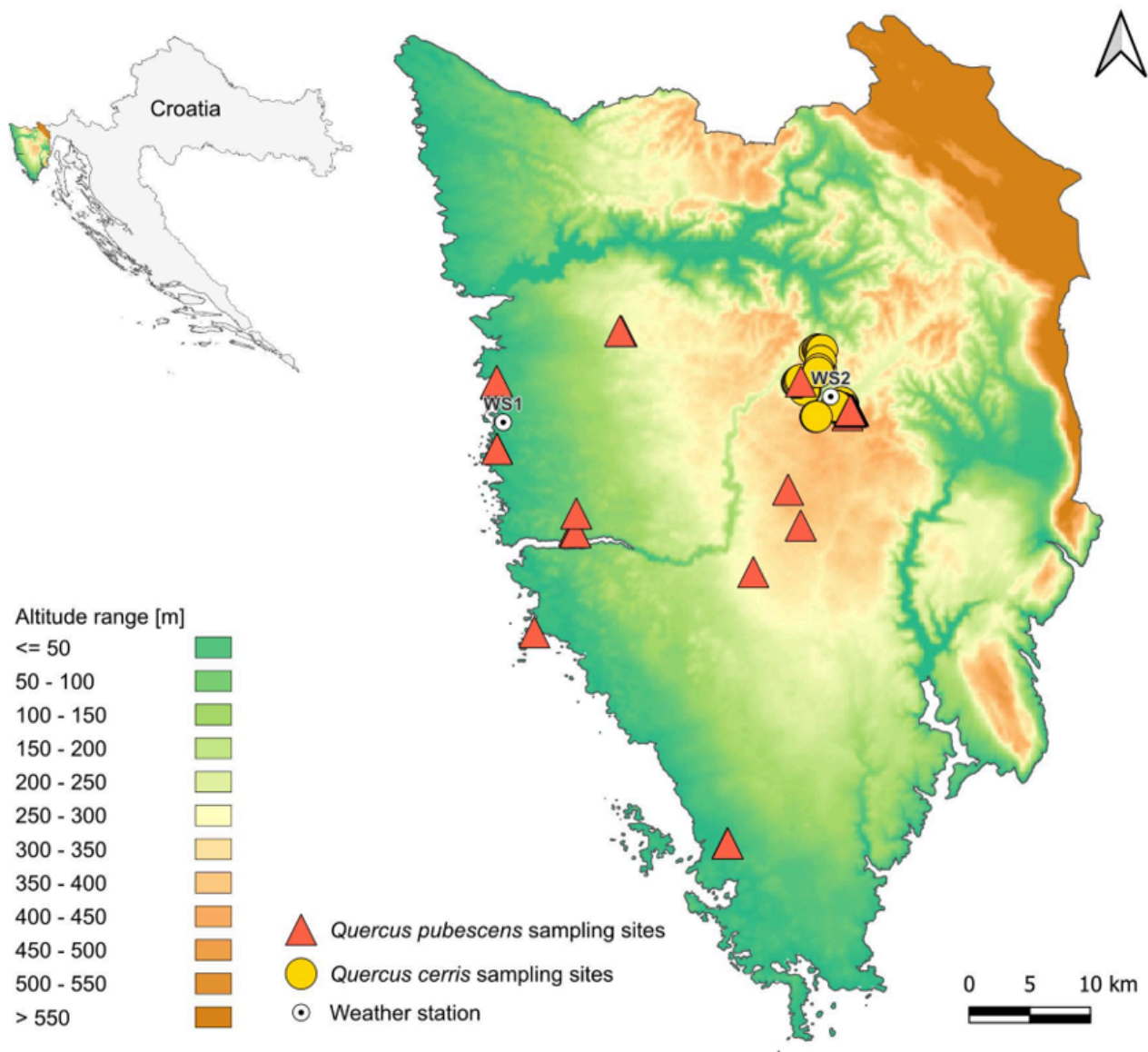


Figure 1

Elevation map of Istrian County with wider soil sampling locations and locations of weather stations (WS1 - Poreč, WS2 - Pazin)

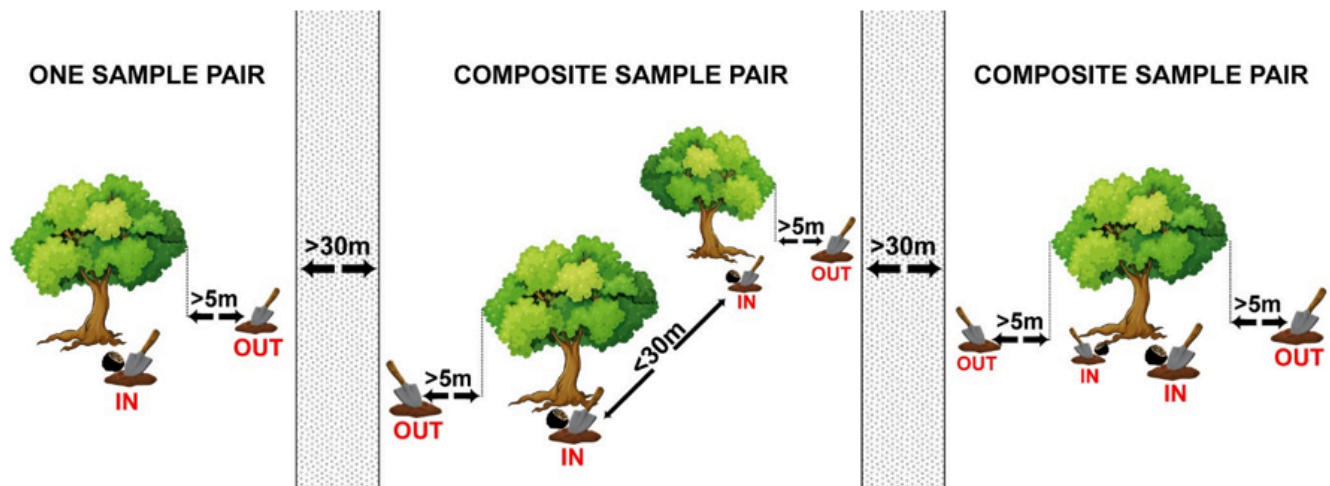


Figure 2

Illustration of sample grouping methodology. Productive sampling zone (IN) and non-productive sampling zone (OUT), each representing one half of the sampling pair, are depicted as a shovel. Composite sample pairs were taken in scenarios where the distance between the found ascocarps or host trees was less than 30 meters, or if multiple *T. aestivum* ascocarps were found beneath one tree

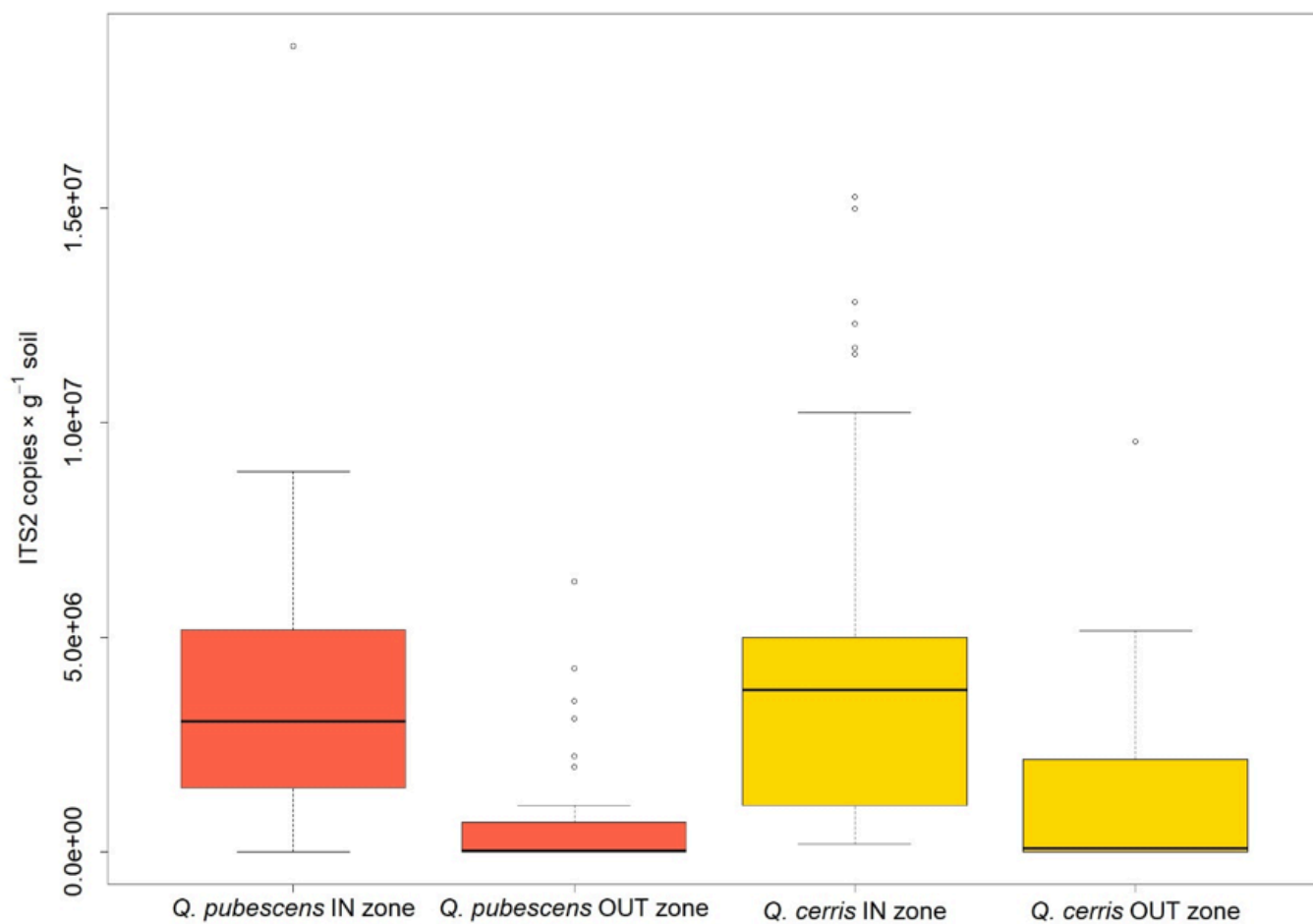


Figure 3

Boxplot visualisation of the mycelial quantity of *T. aestivum* within the research area, divided by host tree species and sampling zone. Red columns - *Q. pubescens* productive (IN) and non-productive (OUT) zones, yellow columns - *Q. cerris* IN and OUT zones

Soil texture triangle of *Q. pubescens* sampling plots

Soil texture triangle of *Q. cerris* sampling plots

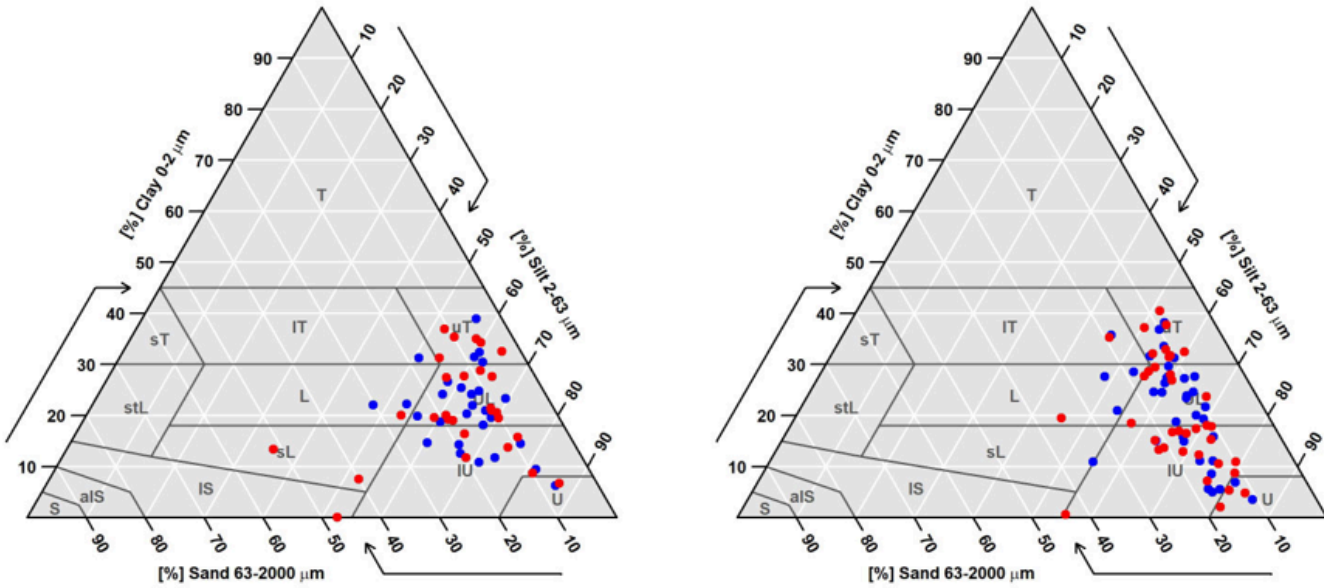


Figure 4

SEA 1974 (DE) soil texture triangles of identified *T. aestivum* productive locations. Blue dots: IN zone. Red dots: OUT zone. L - loam, stL - sandy clay loam, sL - sandy loam, S - sand, aIS - slightly loamy sand, IS - loamy sand, T - clay, uT - silty clay, IT - loamy clay, sT - sandy clay, U - silt, UL - silt loam, IU - loamy silt

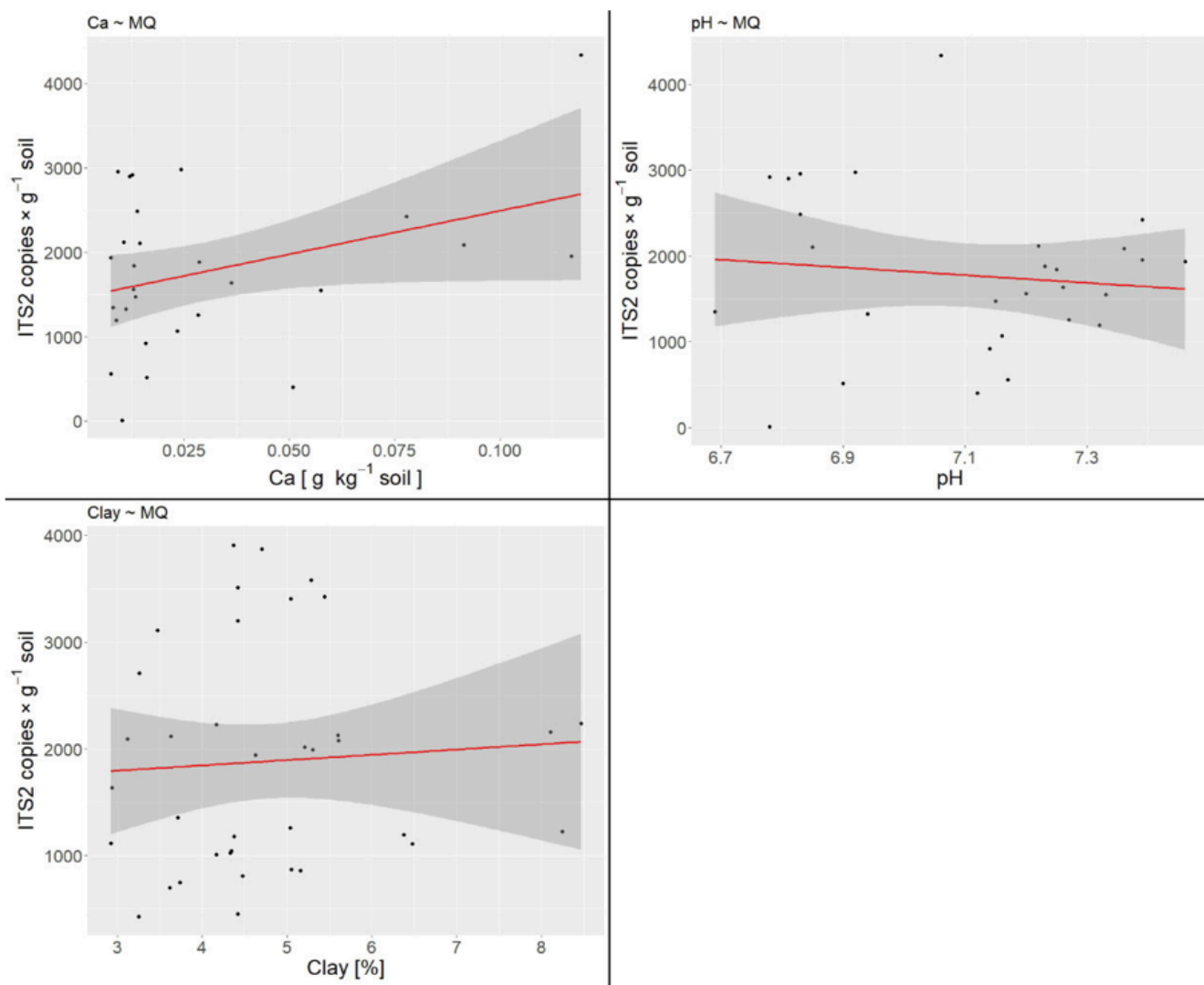


Figure 5

Partial regression graphs of influences of (top left) calcium and (top right) soil alkalinity towards *T. aestivum* mycelium quantity within *Q. pubescens* IN sampling zones, and (bottom left) clay towards mycelium quantity in *Q. cerris* IN sampling zones. Mycelium quantity (MQ) is expressed as the number of ITS2 copies $\times$ g⁻¹ soil

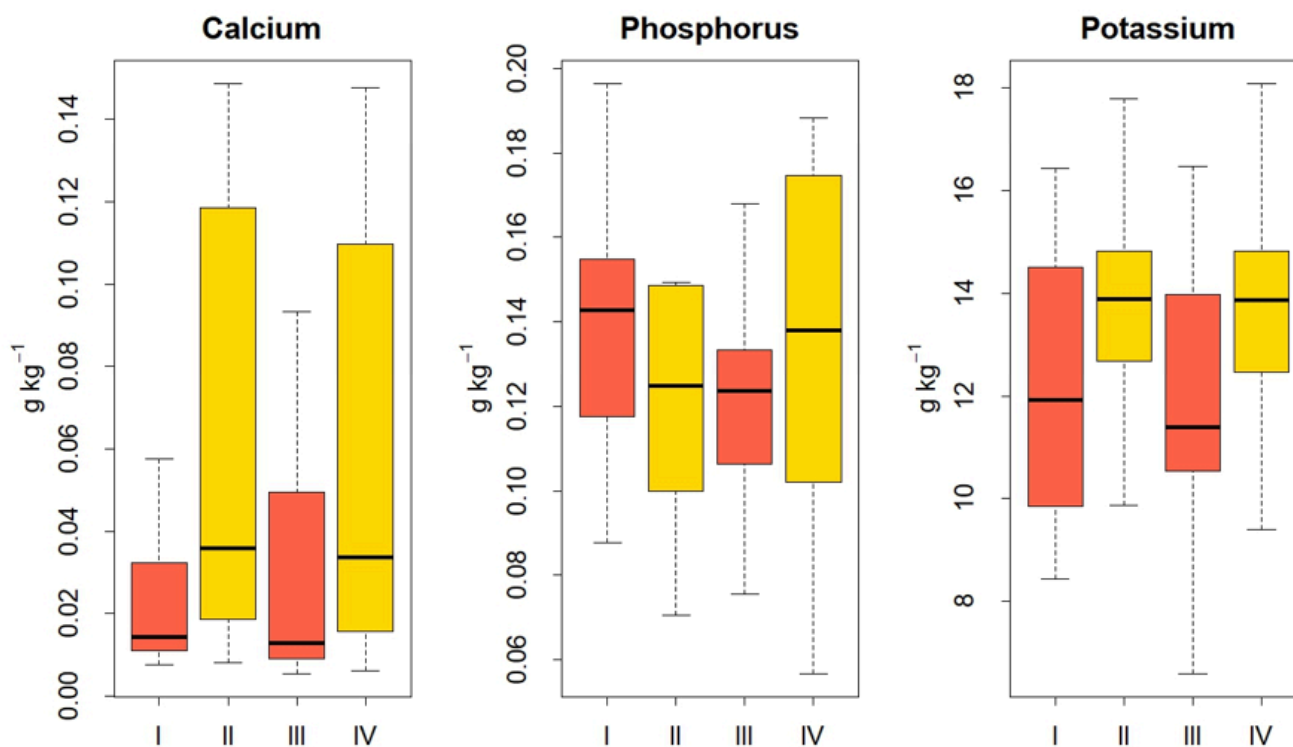


Figure 6

Boxplot visualisation of differences in calcium, phosphorus and potassium amounts (g kg^{-1} soil) between host tree species and sampling zones, respectively. *Q. pubescens* IN zones (I), and OUT zones (III) are depicted in red colour. *Q. cerris* IN zones (II), and OUT zones (IV) are depicted in yellow colour. For better visualisation, values outside whiskers are not included

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