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Do morphological hillslope features affect soil properties and processes promoting chestnut ink disease? The study case of the Northern Apennine mountains

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Abstract. Ink disease caused by the soil-borne Phytophthora cambivora and Phytophthora cinnamomi is threatening sweet chestnut (Castanea sativa) groves in Europe. This study aims to explore whether soil morphology and its related properties influence the development of chestnut ink disease considering the whole soil depth. In C. sativa stand in Northern Italy, along a small altitudinal transect, soil profiles were dug close to ink diseased plants (INK1 at 978 m a.s.l.) and healthy plants (INK2 988 m a.s.l. and INK3 at 998 m a.s.l.) and each soil horizon evaluated for its properties. Further, INK1, INK2 and INK3 had a slope of 3, 9 and 30 %, respectively. The results showed that the lower slope position of INK1 combined with the lower slope gradient than INK2 and INK3 might have promoted the transport of clay particles and water from the latters to the former. Such process allowed the accumulation of clay within the whole INK1 soil profile increasing the saturated hydraulic conductivity and the wilting point. Such soil features might promote the water accumulation within the deeper soil horizons of INK1 which would explain the presence of Phytophthora spp. DNA. The presence of the root pathogen in INK1 might have affected the microbial functionality as observed by the higher abundance of the contact and medium-distance exploration ectomycorrhizal fungal community than the long-distance types. Finally, such study highlighted the pivotal role of soil processes (i.e., clay and water transport) to shape the soil microbial community and soil-borne pathogens because of the changes of edaphic properties.

1 Introduction

Since the end of World War II, forested areas in Italy have been steadily increasing due to reforestation efforts and, more significantly, the abandonment of agricultural and pasture lands (Corona et al., 2008). Today, forests cover more than 37% of the Italian national territory. Chestnut trees (*Castanea sativa* Mill.), which thrive in hilly and mountainous regions, grow at elevations between 250 and 1000 meters above sea level. In the 1950s, the ISTAT inventory reported 447,000 hectares of fruit chestnut groves and 275,186 hectares of coppice chestnut groves (Venanzi et al., 2016). Currently, fruit chestnut groves have dwindled to 147,000 hectares, while coppice chestnut forests now span approximately 600,000 hectares. However, these forests are often aged and abandoned due to socio-economic shifts, policy changes, and phytosanitary issues

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(Fernandes et al., 2022; Venanzi et al., 2016). At the European level, chestnut forests are recognized as habitats of Community interest (9260 Castanea sativa forest) (EC, 2007).

Despite efforts from European and local rural development programs to restore chestnut groves (Buckley, 2020; Gullino et al., 2020), their success has been limited, often hindered by fragmented land ownership and a lack of generational turnover among farmers.

Unmanaged chestnut forests are increasingly vulnerable to emerging phytosanitary threats such as chestnut blight (*Cryphonectria parasitica*), ink disease (*Phytophtora cambivora*), brown rot (*Gnomoniopsis castanea*), and the Asian chestnut gall (*Dryocosmus kuriphilus*) (Maresi et al., 2013; Murolo et al., 2022; Prospero et al., 2023). One of the most severe threats is ink disease, which causes canopy decline, yellowing leaves, branch death, and ultimately the rapid desiccation of the entire tree. The disease can sometimes take years to fully manifest (Prospero et al., 2023), and is primarily caused by *Phytophthora cambivora* (Petri) Buisman and *Phytophthora cinnamomi* Rands in Europe, though other species have also been identified (Jung et al., 2018; Prospero et al., 2023).

Climate change, characterized by increased summer droughts, milder winters, and rising temperatures, has exacerbated ink disease since the early 2000s, creating stressful conditions that make chestnut trees more susceptible to infection (Prospero et al., 2023; Vettraino et al., 2005). The consequences of ink disease are devastating, impacting not only the survival of trees but also the landscape, ecosystem services, and local economies dependent on chestnut yields.

Soil conditions significantly influence both the severity of pathogenic attacks and the resilience of the soil-plant system (Jung et al., 2018). Environmental factors like heavy rainfall, heatwaves, and drought further promote the spread of ink disease (Brasier et al., 1993; Davison, 1988; Jung and Blaschke, 1996; Jung et al., 2013). In many cases, the symptoms of ink disease may only become evident decades after initial infection (Ribeiro, 1978; Tsao, 1990). However, recent studies in the northern Apennines suggest that some chestnut stands are managing to coexist with *Phytophthora* spp., hinting at a potential equilibrium between the pathogen, host, environmental conditions and soil microbiota (Marzocchi et al., 2024).

While most soil microbiology research focuses on the upper soil layers (up to 30 cm deep), where microbial activity is highest, relatively little is known about the subsoil microbiome, despite its importance (Jiao et al., 2018). Microorganisms play a critical role in soil processes like organic matter decomposition, nutrient cycling, and maintaining soil structure (Mendes et al., 2011; Piotrowska-Długosz et al., 2022). They also serve as indicators of soil health (Banerjee and van der Heijden, 2023; Kirk et al., 2004). Moreover, microorganisms are key producers of soil enzymes that regulate organic decomposition and nutrient cycles (Burns et al., 2013; Zuccarini et al., 2020). These enzymes respond quickly to changes in pedoclimatic conditions (Fanin et al., 2022), making them valuable for assessing soil degradation and recovery processes (Bhaduri et al., 2022; Vázquez et al., 2020).

This study aims to investigate how soil morphology, considered soil forming factor, influences: 1) the development of chestnut ink disease, and 2) the Oomycota, fungal and bacterial communities in both topsoil and subsoil horizons.

The study employs an integrated approach, combining soil physicochemical analyses, microbial DNA metabarcoding, and enzyme activity assessments, offering a holistic view of the soil-plant-pathogen system.

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2. Materials and Methods

2.1 Study area and experimental design

The study area is within the municipality of Alto Reno Terme in the Apennine mountains of the province of Bologna (northern Italy), near the border between the Emilia-Romagna and Tuscany regions (Fig 1). The area under investigation is predominantly characterized by fruit chestnut groves, including many centuries-old trees, which represent the primary land use. Chestnut trees have historically dominated this landscape, as highlighted by activities conducted within the National Rural Network program (2014-2020) (Rete Rurale Nazionale, 2023).

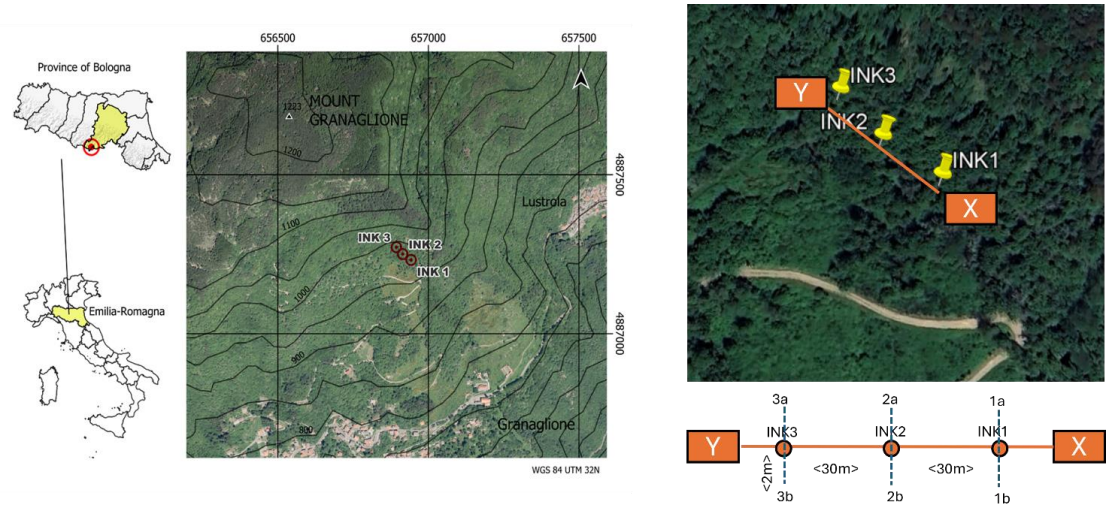

Fig 1. Location of the study area (left) and soil sampling design (right). The X-Y segment represents the sampling transect, where INK1, INK2 and INK3 identify the soil profiles, whereas 1a, 1b, 2a, 2b, 3a and 3b represent the soil sampling points performed by auger to identify the variability of the horizons of the investigated areas. (Image sources: AGEA: © 2020 AGEA; Google Earth: Image © 2025 Airbus)

Geologically, the area features dormant landslide deposits that overlay the turbiditic arenaceous-pelitic member of the Castiglione dei Pepoli formation (Vittori Antisari et al., 2023). The climate is defined by warm summers and moderately cold winters, with a mean annual air temperature of 10.9°C. Seasonal variations range from an average of 20.7°C in August to 2.5°C in January (Fig C1). The area receives an average annual precipitation of 1,647 mm, unevenly distributed throughout the year, with the heaviest rainfall occurring in winter, spring, and fall, particularly in November. There is no defined arid period.

An abandoned chestnut grove on the south-facing slope of Mount Granaglione was selected as the study site. The grove spanned an elevation range from 970 to 1,050 meters above sea level (a.s.l.). The abandoned chestnut grove had old chestnut trees (> 60 years old) with an average plant spacing of 10 m, unmanaged since 2008. The understory was overgrown of vegetation mainly composed of fern plants. Three specific areas were chosen along an elevation transect between 978 and 998 m a.s.l., with 10-meter elevation intervals. This transect included the areas INK1 at 978 m a.s.l. and

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with a gentle slope (3 %), INK2 at 988 m a.s.l. and with moderate slope (9 %), and INK3 at 998 m a.s.l. and with a steep slope (30 %) (Fig 1 and Fig 2).

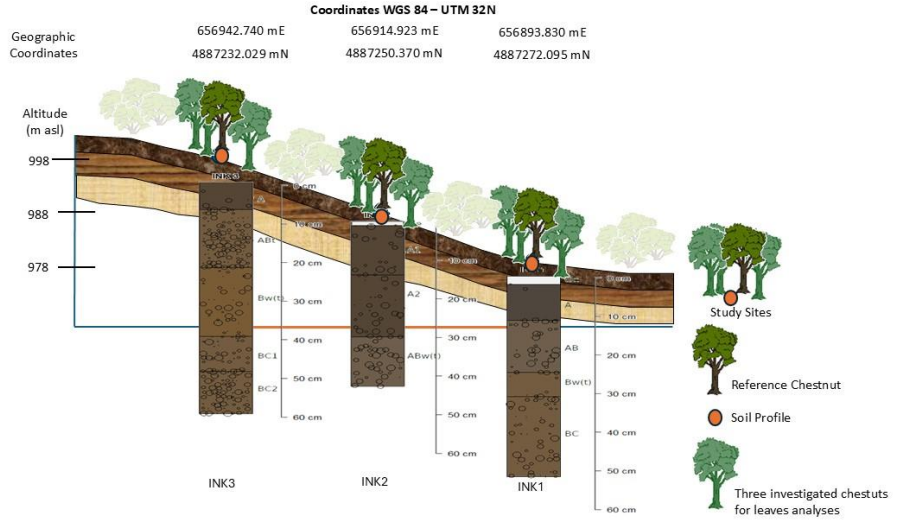

Fig 2. Scheme of the soil profiles and their position along the mountain slope.

The experimental design was carried out along a 60 m transect, with three soil profiles (designated INK1, INK2, and INK3) spaced 30 m apart to assess changes in soil properties between symptomatic and asymptomatic chestnut tree sites. Each soil profile was opened 1 m downhill from a reference tree at each site. Additionally, two supplementary auger samples (labeled 'a' and 'b') were collected 2 m from each soil profile to capture the variability in horizons across the different areas studied (Fig 1). Both the soil profiles and the soil horizons sampled using an auger were described following the guidelines of Schoeneberger et al. (2012). A representation of the soil profiles is shown in Fig 2.

We are aware that a single chestnut grove may limit the generalizability of findings to other regions or soil types. However, soil has high heterogeneity even at small spatial scales (e.g., Cambardella et al., 1994; Pätzold et al., 2008) because even the slightest difference in soil forming factors could heavily affect the type and intensity of the pedogenetic processes (Türkeş et al., 2023). For example, it is recognized that the vertical and horizontal transport of water in soil may be different along the hillslope also at the microtopography level (Liu et al., 2022; Thompson et al., 2010). In addition, mountain areas are characterized by a huge topographic variability boosting soil diversity (Oueslati et al., 2013; Seibert et al., 2007; Vittori Antisari et al., 2023). Since the importance of the pedogenic features in shaping soil microbial community (Delgado-Baquerizo et al., 2020; Leewis et al., 2022), soil sampling in other chestnut groves might a wider microbial dataset, but it might prevent to understand the relationship between soil properties/processes with microbial community.

Approximately 500 g of soil was collected from each genetic horizon for physicochemical and biochemical analysis. For metabarcoding analyses, soil from each genetic horizon was sampled using a sterile 5 cm diameter core sampler. The samples were then stored in a refrigerated box and transported to the laboratory.

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The chestnut trees in INK1 exhibited signs of decline, including thinned crowns, smaller chlorotic leaves, dead branches, and dark, flame-shaped lesions on the bark at the root collar. These lesions, extending to the base of the trunk and visible after bark removal, are characteristic of *Phytophthora* infection. In contrast, the trees in INK2 and INK3 showed no such symptoms and appeared healthy. (Fig C2).

Three reference trees were selected close to each soil profile to assess their nutrient status by sampling mature leaves from the mid-branches analyzed according to the method of Vittori Antisari et al. (2018). In summary, leaves from symptomatic trees (INK1) showed lower concentrations of nitrogen, potassium, calcium, and boron compared to healthy trees, while healthy trees exhibited lower manganese content than symptomatic ones (Table 1).

Table 1. Mean (n = 4) nutrient content in leaves of the chestnut trees located close to soil profiles INK1, INK2 INK3. Different letters indicate significant differences among the three sites ($p \leq 0.05$) according to Kruskal-Wallis's test.

Nutrient		INK1	INK2	INK3
N	%	2.1a	2.5b	2.7c
Al	g kg^{-1}	0.12	0.21	0.12
Fe	g kg^{-1}	0.15	0.27	0.16
Mn	g kg^{-1}	0.41a	0.27b	0.27b
K	g kg^{-1}	7.1	7.8	10.7
Ca	g kg^{-1}	6.8	7.6	10.1
Mg	g kg^{-1}	2.2	2.0	2.0
P	g kg^{-1}	1.8a	1.6c	1.7b
B	mg kg^{-1}	2.7b	20.2a	20.2a

2.2 Soil physicochemical analyses

The pH was determined in 1:2.5 soil:water suspension (w:v) after two hours of shaking and using a pH-meter (Crison). Total organic carbon (OC) and total nitrogen (TN) were detected by Dumas combustion with CHN elemental analyser (Flash 2000, Thermo Fisher Scientific, USA). The particle-size distribution was determined by the pipette method (Gee and Bauder, 1986). Additional details on soil analyses can be found in the Appendix A.

2.3 Soil biochemical and enzyme analyses

Microbial biomass carbon (Cmic), microbial biomass nitrogen (Nmic) and the basal respiration (BR) were measured following the methodology of De Feudis et al. (2020), with detailed descriptions provided in the Supplementary materials.

The activities of β-cellobiohydrolase (EC 3.2.1.91), α-glucosidase (EC 3.2.1.21), β-glucosidase (EC 3.2.1.20), and xylosidase (EC 3.2.2.27), with their respective substrates being 4-MUF-β-D-cellobioside, 4-MUF-β-D-glucoside, 4-MUF-β-D-glucoside, and 4-MUF-7-β-D-xyloside were assessed using the methods described by Marx et al. (2001) and Vepsäläinen

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et al. (2001). A moist soil sample (equivalent to 1 g of oven-dried material) was placed into a sterile jar, and 50 ml of Na-acetate buffer (pH 5.5) was added. The suspension was homogenized using an UltraTurrax at 9,600 rpm for 3 minutes. From this suspension, 100 µl aliquots were dispensed into a 96-well microplate, with three analytical replicates per sample per substrate. Then, 100 µl of 1 mM substrate solution was added to achieve a final substrate concentration of 500 µM. Fluorescence (excitation 360 nm; emission 450 nm) was measured using a Fluoroskan Ascent automated plate reader (Thermo Fisher Scientific) at intervals of 0, 30, 60, 120, and 180 minutes, with incubation at 30°C.

The enzyme activities recorded were normalized by microbial biomass (Cmic) and organic carbon (OC) content to calculate microbial biomass-specific and organic C-specific enzyme activities, allowing for reliable comparisons of enzyme activity across the different soil profiles.

2.4 DNA extraction and taxonomic assignment

Total DNA was extracted from the soil samples using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, with three technical replicates performed for each sample. For microbial and fungal community analysis, we amplified the 16S rDNA V3-V4 region using the Polymerase Chain Reaction (PCR) primers 16S 341F/805R (García-López et al., 2023) and the Internal Transcribed Spacer (ITS) region using primers ITS1F/ITS4R (Gardes and Bruns, 1993; White et al., 1990) respectively. The PCR products included flow-cell binding domains and unique indices for sequencing.

Libraries were sequenced on MiSeq instrument (Illumina, San Diego, CA) using 300-bp paired-end mode. Base calling, demultiplexing, and adapter masking were performed with Illumina BCL Convert v3.9.3 (https://emea.support.illumina.com/). Reads were filtered and trimmed using Trimmomatic v.0.39, and read quality was assessed with FastQC. The resulting FASTQ sequences were analyzed using Kraken 2, which compares k-mers from sequencing reads to those from the Silva ribosomal RNA Database (release 138.1) (Wood et al., 2019). Taxonomic abundance was estimated using Bracken (Lu et al., 2017).

Microbial communities (bacteria and fungi) were analyzed at the phylum level. Fungal communities were further analyzed at the genus level to identify fungal guilds and explore ectomycorrhizal guilds using FungalTraits (Põlme et al., 2020).

Additionally, all sampled genetic horizons were tested for *Phytophthora* detection following the protocol by Burgess et al. (2022) with P4 primer pair. These primers were first validated on a positive DNA control (*Phytophthora cambivora*) to ensure they produced a 250 bp amplicon, visible on an agarose gel. Total DNA from each soil sample was normalized to 2 ng/µL and used as the template for the initial PCR. The amplicons from this round served as templates for a nested PCR.

It is important to mention that we are aware that the relic DNA constitutes 20–80% of microbial DNA (Carini et al., 2017), and its persistence time can range from weeks to years, depending on soil characteristics (e.g., minerals and humic substances) (Levy-Booth et al., 2007). In our study, we preferred to analyze the whole DNA, without removing relic DNA (Carini et al., 2017; Wang et al., 2025), in order to obtain an overview of all the living and dead microorganisms that may have contributed to disease development under these specific environmental conditions.

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2.5 Indices calculation and statistical analyses

The hydraulic properties of the soil, including water content at saturation (THS), field capacity at -330 cm matric potential (FC), wilting point at -15,000 cm (WP), and saturated hydraulic conductivity (KS), were determined for each horizon using the Web interface for European hydraulic pedotransfer functions (https://ptfinterface.rissac.hu/app_direct/ptf/, Szabó et al., 2021). The input variables included horizon depth, organic carbon content, bulk density, and particle size distribution. Available water capacity (AWC) was calculated as the difference between FC and WP. THS, FC, WP, and AWC were adjusted for skeletal content by multiplying by (100 - Sk)/100, where Sk represents the coarse fragment content in percent. KS was also corrected following the method described by Naseri et al. (2020), using an m value of 1.8 (the mean between spherical and oblate fragments with an aspect ratio of 5).

The Synthetic Enzymatic Index (SEI) a composite measure of microbial functional capacity, was calculated as the sum of all soil enzyme activities, as described by Vittori Antisari et al. (2018) and was expressed per unit of soil total organic C (SEI/OC) and/or per unit of soil microbial biomass C (SEI/Cmic)

To evaluate differences in physico-chemical and biological variables among soil profiles in study sites, a Kruskal-Wallis test was conducted after assessing data heteroscedasticity. This analysis was performed in RStudio (R version 4.4.1) using the *agricolae* package

3 Results

3.1 Soil classification, physicochemical and hydraulic properties of soils

The investigated soil profiles were classified as Luvisols according to the World Reference Base for Soil Resources (IUSS Working Group WRB, 2022). Specifically, INK1 was categorized as Skeletic Luvisol (loamic, epidystric), INK2 as Leptic Skeletic Luvisol (loamic), and INK3 as Skeletic Luvisol (loamic), reflecting the significant presence of coarse fragments throughout the profiles (20%, 22%, and 44% for INK1, INK2, and INK3, respectively). According to the Soil Survey Staff classification system (Soil Survey Staff, 2022), the clay illuviation process was evident in INK2 and INK3, which were classified as Lithic Hapludalf (loamy-skeletal, mesic) and Lithic Hapludalf (loamy-skeletal, mesic, truncated), respectively. INK1, classified as Ruptic-Alfic Dystrudept, exhibited cambic horizons with weak argillic features. Its dystric properties were indicated by a low pH (5.6) and base saturation percentage (34.0%).

The physicochemical and hydraulic properties of the soil profiles are detailed in Tables B1 and B2. Generally, the soil texture across all horizons was silty loam, except for the Bw(t) horizon in INK1, which had a loamy texture. Kruskal-Wallis test results (Fig. 3) showed that pH and base saturation in INK1 (5.6 and 33.9%, respectively) were significantly lower compared to INK2 (6.5 and 47.3%, respectively) and INK3 (6.6 and 51.4%, respectively). The clay content in INK1 (218 g kg^{-1}) did not show significant differences, likely due to high variability within the profile (Fig 3).

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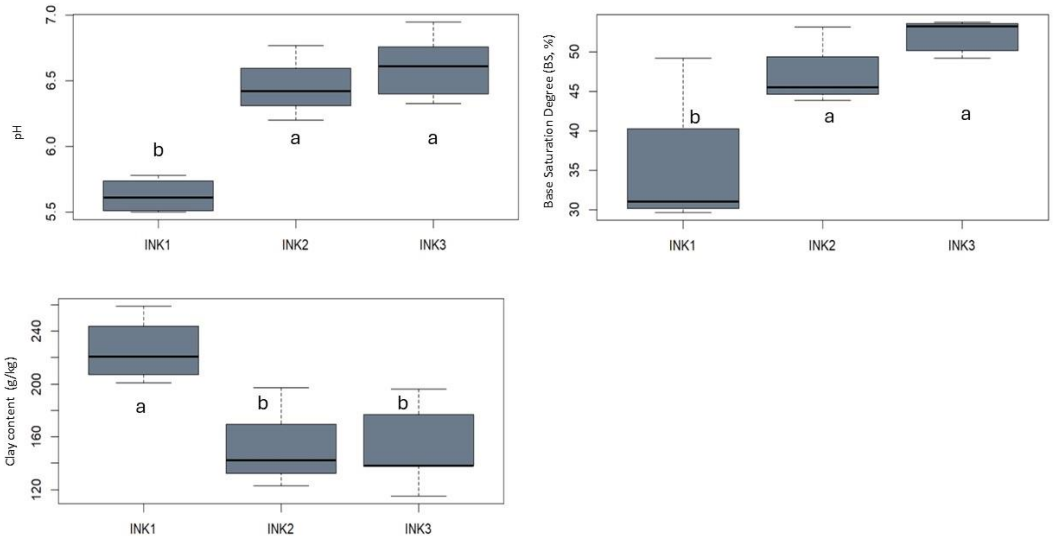

Fig 3. Boxplots showing the pH, base saturation degree and clay content in soil under ill chestnut plants (INK1) and healthy chestnut plants (INK2 and INK3). Different letters indicate significant differences ($p \leq 0.05$) according to Kruskal-Wallis's test.

Due to the high variability in water-related soil properties within each profile, few statistically significant differences were observed. Notable differences were found in the wilting point (WP), which was higher in INK1 (14.9%) compared to INK2 (11.7%) and INK3 (8.2%), and in saturated hydraulic conductivity (KS), which was higher in INK1 (25.1%) compared to INK2 (4.6%) and INK3 (6.5%) (Fig 4).

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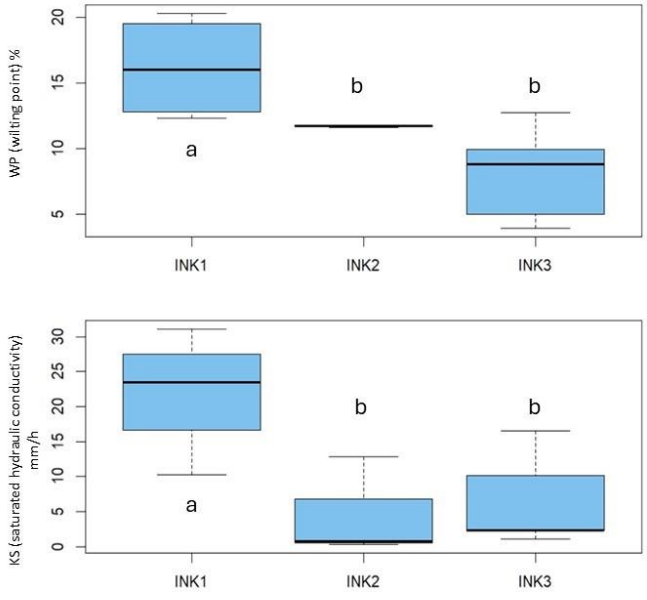

Fig 4. Boxplot showing the permanent wilting point and the saturated hydraulic conductivity in soil under ill chestnut plants (INK1) and healthy chestnut plants (INK2 and INK3). Different letters indicate significant differences ($p \leq 0.05$) according to Kruskal-Wallis's test.

3.2 Soil organic matter, related properties and enzyme activities

As expected, most of the parameters related to soil organic matter content (Table B3) showed the highest values in the A horizon and decreased with depth. No significant differences were found among the different parameters investigated for C and N cycle. Only water–extractable organic C (WEOC) content and WEOC:water–extractable N (WEN) ratio showed significant differences among the sites, where higher values were found in INK1 compared to INK2 and INK3 (Fig 5).

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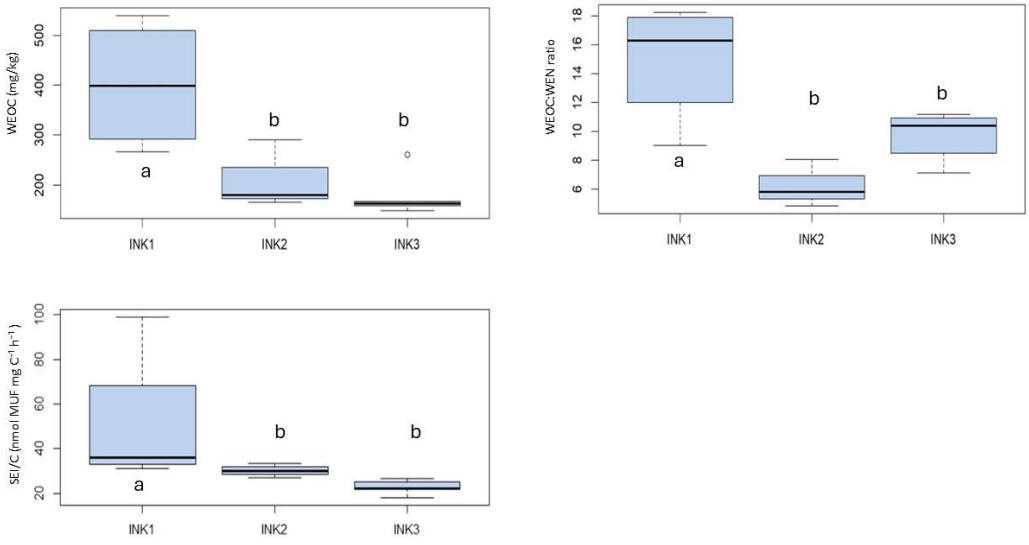

Fig 5. Boxplot showing WEOC content, the WEOC:WEN ratio and the SEI/C of C and N parameters and specific Synthetic Enzymes Index (SEI/OC) in soil under ill chestnut plants (INK1) and healthy chestnut plants (INK2 and INK3). Different letters indicate significant differences ($p \leq 0.05$) according to Kruskal-Wallis's test. WEOC: water-extractable organic carbon; WEN: water-extractable nitrogen; SEI/OC: Synthetic Enzymatic Index expressed per unit of soil total organic C.

Across the entire soil profiles, enzyme activities and calculated indices did not show significant differences among the study sites (Table B4), mainly due to the high variability within the soil profiles. However, evaluating the pedogenetic horizons, both organic carbon-specific and microbial biomass-specific enzyme activities were highest in the Bw(t) horizon of INK1 (Figure C3). Although elevated specific activities were observed in the AB and Bw(t) horizons of INK1, the Synthetic Enzymatic Index (SEI/OC) for INK1 was significantly higher than in INK2 and INK3, with average values of 50.5 nmol MUF mg C^{-1} h^{-1} compared to 30.1 and 22.7 nmol MUF mg C^{-1} h^{-1}, respectively (Kruscall-Wallis test $\alpha = 0.05$, p.chisq = 0.01) (Fig 5).

3.3 Biodiversity of the fungal and bacterial communities

Fig 6 presents the results of the metagenomic analyses for bacterial communities, showing the percentage attributed to the principal phyla across different soil horizons. Acidobacteriota and Proteobacteria were the predominant phyla identified. Proteobacteria percentages were relatively stable across all samples, whereas Acidobacteriota showed notable variability. Specifically, while the prevalence of Acidobacteriota was comparable in INK2 and INK3, it significantly increased in INK1, particularly in the AB horizon. Minor phyla generally decreased in proportion to the rise in Acidobacteriota. Additionally, Nitrospirota, a group of nitrifying bacteria, were present in significant proportions (up to 7%) in some deeper horizons of INK2 and INK3 (e.g., INK2 Bw(t) and INK3 BC2), whereas their presence was minimal in INK1.

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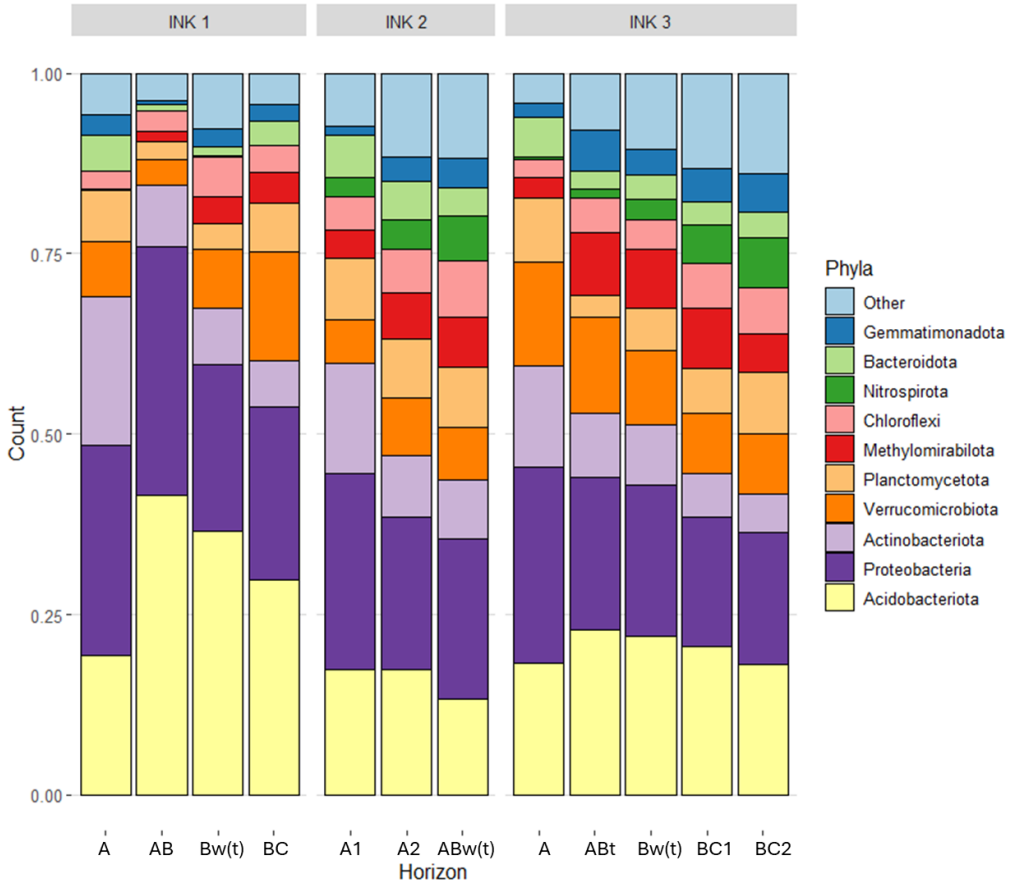

Fig 6. Distribution of bacterial phyla in the horizons of soil profiles under ill chestnut plants (INK1) and healthy chestnut plants (INK2 and INK3). Phyla with maximum count < 5% are grouped in "Other".

Phytophthora was detected only in the soil from INK1, where a faint band was observed in samples belonging to the BC horizon.

The fungal composition at the phylum level exhibited greater diversity across the profiles compared to bacterial phyla. As shown in Fig 7, the uppermost horizons of all soil profiles displayed distinct fungal communities compared to the subsoil horizons. In the uppermost horizons, there was a relatively balanced distribution among Basidiomycota, Ascomycota, and Mortierellomycota. In contrast, in the deeper horizons, Basidiomycota emerged as the predominant phylum.

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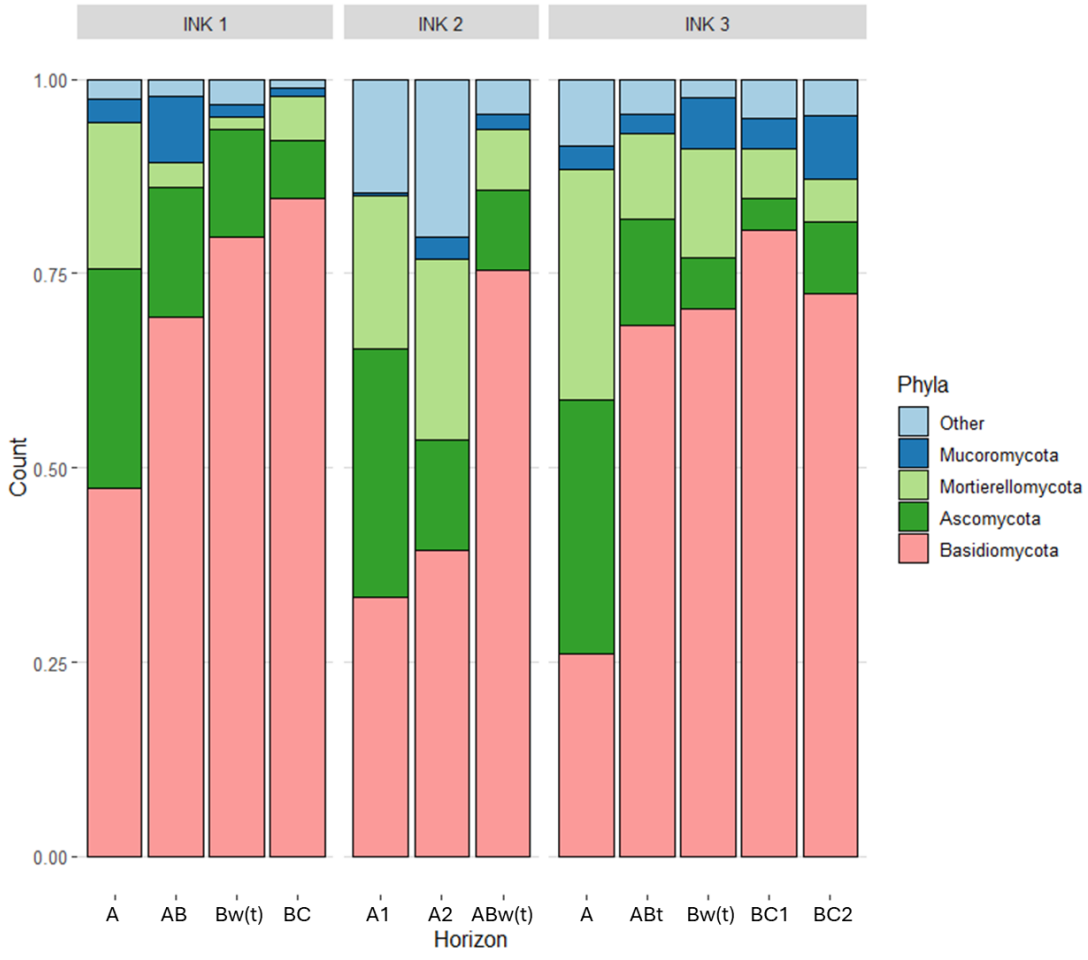

Fig 7. Distribution of fungal phyla in the horizons of soil profiles under ill chestnut plants (INK1) and healthy chestnut plants (INK2 and INK3). Phyla with maximum count < 5% are grouped in "Other".

Fungal composition was further analyzed at the genus level to better understand fungal trophism (Fig 8). In the mineral horizons of INK1, ectomycorrhizal fungi predominated, comprising between 36% and 82% of the fungal community, with notable genera including *Russula*, *Lactarius*, and *Lactifluus* (Figure C3). INK3, on the other hand, was dominated by soil saprotrophs, which made up between 39% and 82% of the fungal community, with key genera such as *Geminibasidium* and *Saitozyma*. INK2 displayed a mixed composition: the topsoil was primarily dominated by ectomycorrhizal fungi (48%), while the subsoil was characterized by a higher prevalence of soil saprotrophs (ranging from 40% to 64%).

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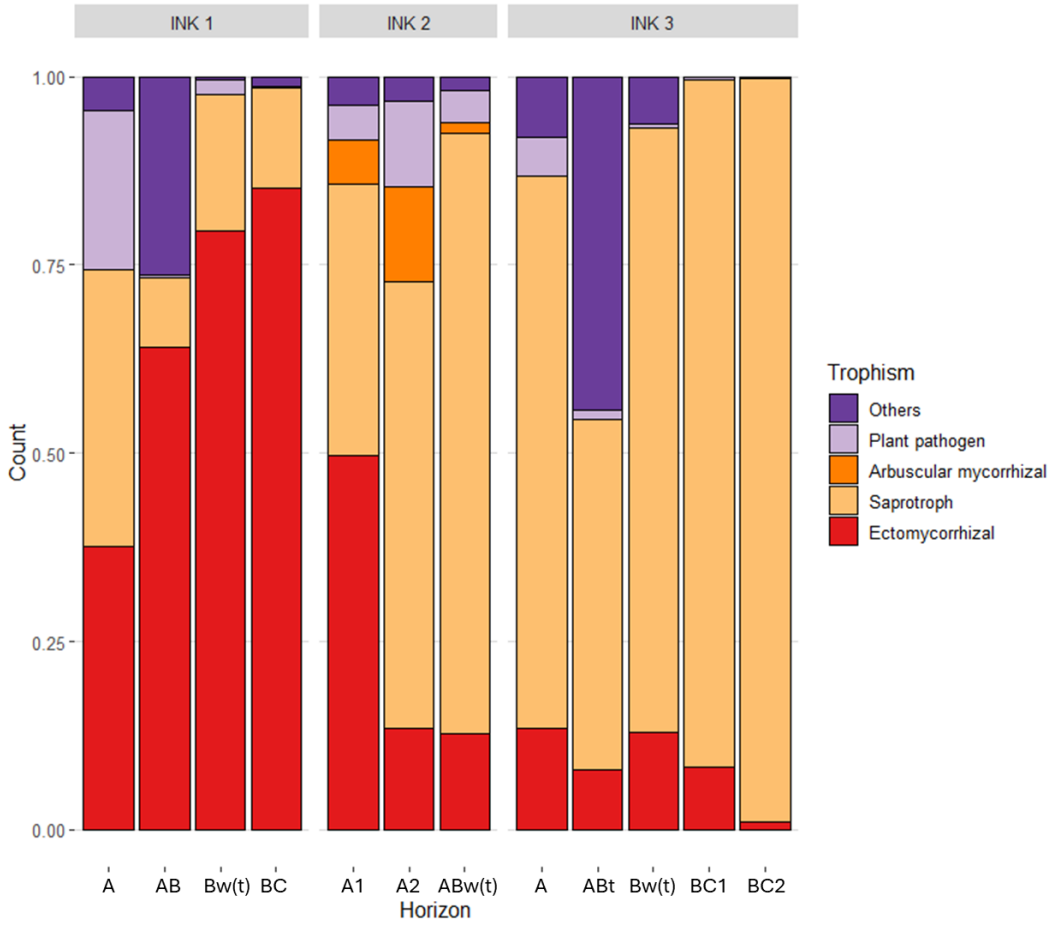

Fig 8. Distribution of fungal trophism, calculated on genus, in the horizons of soil profiles under ill chestnut plants (INK1) and healthy chestnut plants (INK2 and INK3).

An interesting observation was the presence of plant pathogens in the topsoil of INK1, which accounted for up to 20% of the total fungal community, predominantly from the genus *Rhizoctonia*. Similarly, plant pathogens were also notable in the A2 horizons of INK2, where they were mostly from the genus *Bipolaris*.

The distribution of mycorrhizal fungi varied across the soil profiles (Fig 9). In INK1, the mycorrhizal community was predominantly comprised of contact and medium-distance mycorrhizal fungi, while short-distance mycorrhizae were present in low abundance, and long-distance mycorrhizae were not observed. In contrast, both INK2 and INK3 were characterized by a high prevalence of short-distance mycorrhizae.

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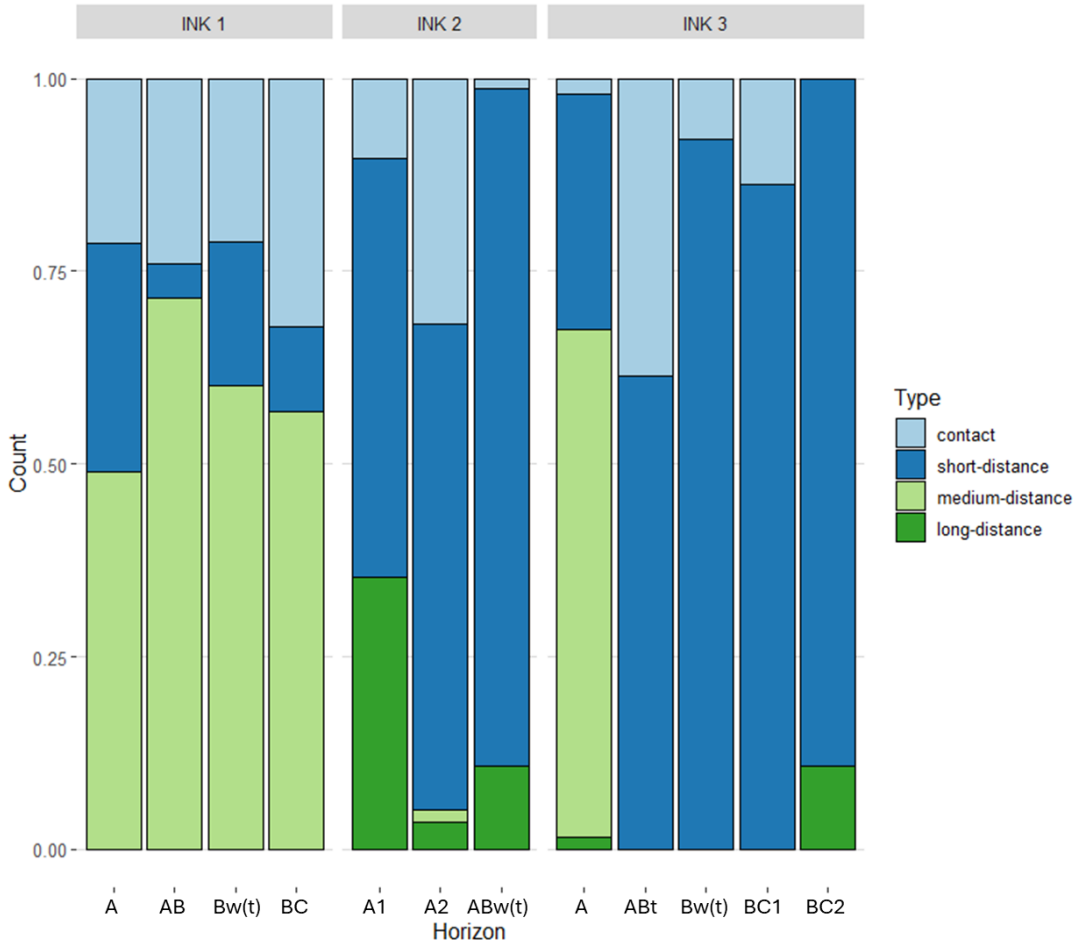

Fig 9. Distribution of fungal mycorrhiza, calculated on genus, in the horizons of soil profiles under ill chestnut plants (INK1) and healthy chestnut plants (INK2 and INK3).

4 Discussion

The investigated soils were classified as Luvisols (IUSS Working Group WRB, 2022), which are characterized by the mobilization and transport of clay, leading to its accumulation in argic horizons. This clay transport, known as lessivage, is driven by the vertical flux of water and is further facilitated by the presence of cracks, pores, sandy-loam texture, and rock fragments (Quénard et al., 2011). Additionally, the hillslope position influences both vertical and horizontal clay transport by water (Krevh et al., 2023). In our study site, we hypothesize that clay particles are transported horizontally both on soil surface and in subsurface horizons from uphill to downhill during rainfall events, resulting in the accumulation of clay in the whole soil profile of INK1 (Fig 10).

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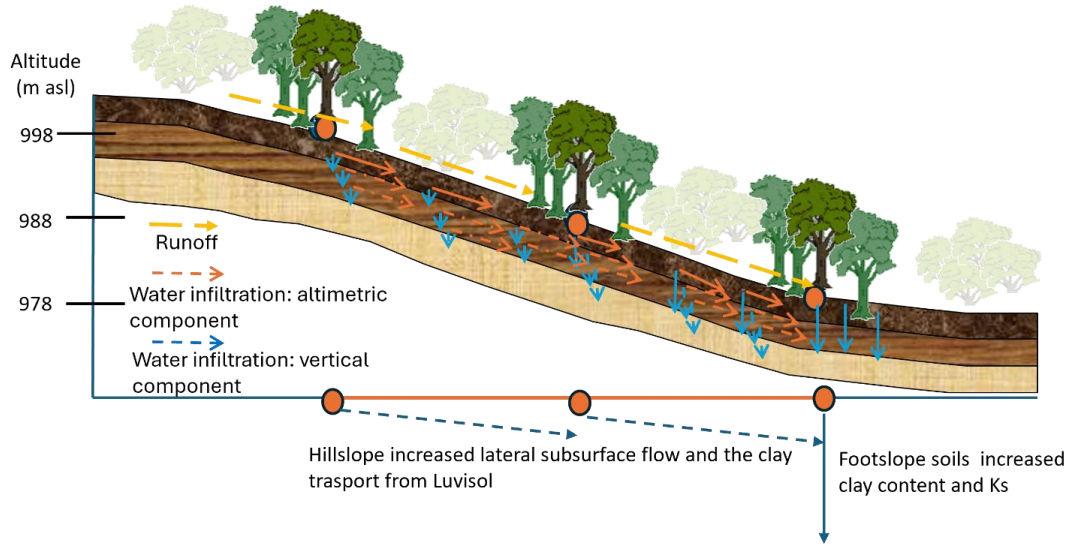

Fig 10. Conceptual figure showing the water infiltration, superficial runoff and horizontal subsurface movement within the study area. KS: saturated hydraulic conductivity.

The presence of downhill water flow toward INK1 is supported by the generally lower saturated hydraulic conductivity (KS) observed in INK2 and INK3, which are situated uphill from INK1 (Zambon et al., 2021). Additionally, the higher slope gradient in INK3 likely enhances the movement of water downhill, particularly contributing to increased water runoff. The elevated runoff rates in INK2 and INK3 are further corroborated by the absence of an organic horizon in INK3 and the shallower organic horizon in INK2 compared to INK1 (Berg and McClaugherty, 2020).

INK1 showed dystric characteristics in the subsoil and its morphological position at the footslope could explain the increased clay content which may have been transported downhill by both runoff process and subsurface flow. This phenomenon may be associated with the high saturated hydraulic conductivity (KS) in INK1 (>23 mm h^{-1}), which allows for easier water infiltration into the subsoil. Increased water infiltration facilitates greater cation leaching, leading to a decline in soil pH (Jobbágy and Jackson, 2003; Slessarev et al., 2016).

The development of *Phytophthora*-related disease symptoms appears to be influenced by hillslope position. The high-water infiltration and clay content in the INK1 soil, evidenced by numerous redox mottles in the AB horizon, suggest consistently moist soil conditions (Doro et al., 2017; Mohanty and Mously, 2000). These conditions are conducive to the survival and proliferation of *Phytophthora*, consistent with findings from other studies (Gyeltshen et al., 2021; Rhoades et al., 2003). Microbial DNA analysis showed the presence of *Phytophthora* in the BC horizon of INK1, despite the generally short persistence of *Phytophthora* DNA in soil, typically lasting only a few days (Kunadiya et al., 2021). The higher WEOC content observed in INK1 may be linked to the presence of infected plants, as pathogen infections are known to influence carbon root exudation rates (Lanoue et al., 2010; Meier et al., 2013; Preece et al., 2024). Pathogen-induced changes in root exudation can elevate WEOC content and alter the carbon-to-nitrogen ratio of the soluble organic fraction. Given that plant tissue characteristics affect soil organic matter quality (Angst et al., 2019), the increased WEOC:WEN ratio in INK1 suggests that disease-affected plants are altering the quality of organic matter reaching the soil. This high WEOC:WEN

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ratio indicates a lower nitrogen availability for both the chestnut plants and the microbial communities. Previous research has shown that pathogen infections can stimulate plants to produce carbon-rich metabolites, such as phenols (Dinis et al., 2011; Lovat and Donnelly, 2019). However, it is also possible that the increased moisture conditions in INK1, due to its higher KS, might have further contributed to the higher WEOC content (Christ and David, 1996; Fröberg et al., 2006; Kaiser et al., 2001). It is known that soil microbial activity is usually stimulated by the presence of available carbon sources (Jansson and Persson, 2015) but the absence of nitrogen input from available organic compounds could promote nitrogen immobilization into the microbial biomass (Jansson and Persson, 2015). In this study, nitrogen deficiency in chestnut trees may be attributed to microbial nitrogen immobilization processes. Additionally, in INK1, a variation in nitrogen cycling was indicated by changes in the soil microbial community, notably the absence of nitrifying taxa like *Nitrospirota*. The low abundance of *Nitrospirota* in the *Phytophthora*-affected soil of INK1 could be linked to reduced nitrogen availability, as these bacteria are highly sensitive to changes in soil pH, nitrogen levels, and other nutrients due to their role in nitrite oxidation and complete ammonia oxidation (D'Angelo et al., 2023). However, the adaptive mechanisms of different *Nitrospira* species and their influence on ecological processes and microbial community assembly remain underexplored. The observed decrease in pH and changes in environmental conditions may contribute to the increased abundance of Acidobacteriota in the deeper horizons of INK1, as these bacteria are well-adapted to such edaphic conditions (Kalam et al., 2020). This finding highlights the importance of evaluating the vertical variability of microbial communities across different soil horizons to gain a more comprehensive understanding of soil functioning (Maitra et al., 2024). While most research to date has focused on microbial communities in topsoil horizons (Naylor et al., 2022) there is increasing recognition of the importance of exploring microbial distribution in deeper soil horizons and across functional spatial scales (Carteron et al., 2021).

Consistent with previous studies (e.g., Dukunde et al., 2019; López-Mondéjar et al., 2015), the bacterial community was primarily dominated by Acidobacteria, Proteobacteria, Verrucomicrobiota, and Actinobacteria. No significant differences in bacterial populations were observed across the sites and soil horizons, except in INK1, where Acidobacteriota were notably more prevalent, particularly in the deeper mineral horizons. The observed changes in soil physicochemical properties, shifts in bacterial communities towards Acidobacteriota, and the presence of *Phytophthora* in the BC horizons of INK1 correlate with increased specific enzyme activities. This increase likely reflects a higher microbial physiological capacity for adaptation rather than a rise in microbial biomass (Cmic) (Marinari et al., 2022). Hydrolytic enzymes are influenced by substrate availability and microbial community composition (Coonan et al., 2020; Qu et al., 2020). The specific activity, i.e. the values of distinct enzymes activity per unit of carbon, allowed comparison among the horizons and the soil profiles (Trasar-Cepeda et al., 2008). The increased specific hydrolytic enzyme activities observed in the deeper horizons of INK1, associated with Phytophthora inoculum and shifts in soil properties, suggest that microbial communities are adapting to these changes by maintaining essential roles in nutrient cycling and potentially establishing new ecological equilibria and functions within the soil-plant-microorganism system. This adaptation helps counterbalance nutrient imbalances and deficiencies. The higher Synthetic Enzymes Index (SEI/OC) values in INK1 could indicate a potential ecological strategy to preserve soil metabolic activity related to nutrient cycling. Elevated SEI/OC in both the AB and Bw(t) horizons points to heightened metabolic activity in the deeper soil layers, where Phytophthora has likely enriched hydrolytic enzymes that target organic compounds. This could reflect a loss or alteration of labile organic matter (Trasar-Cepeda et al., 2008), driven directly by ink disease and indirectly by shifts in microbial communities. Another possible explanation is that these changes

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are linked to shifts in microbial community composition, both in terms of taxa and the functions expressed through enzymatic activities (Nannipieri et al., 2012). Additionally, since fungal communities represent a significant portion of soil microbial populations and are key players in the decomposition of soil organic matter (Frey et al., 2014), they may have a more profound influence on soil enzyme activities than bacteria. In fact, we observed notable shifts in fungal trophism and mycorrhizal exploration strategies in INK1, further emphasizing the role of fungi in shaping soil metabolic processes.

Our findings align with previous studies indicating a positive correlation between soil moisture and ectomycorrhizal growth (Castaño et al., 2017; Kilpeläinen et al., 2017). The increased water infiltration potential in INK1 not only facilitated *Phytophthora* infection in chestnut plants but also created a more favorable environment for ectomycorrhizal fungi in the horizons below the A horizon of INK1. Furthermore, ectomycorrhizal fungi have been shown to offer protection against *Phytophthora* infections (Branzanti et al., 1999; Corcobado et al., 2014; Scattolin et al., 2012), suggesting that their increased presence in INK1 could be a response to the disease.

We also observed that the variation in mycorrhizal exploration types along the INK1 profiles could be attributed to changes in the physicochemical and biochemical properties of the soil. The ectomycorrhizal community, influenced by soil water availability, showed a higher abundance of contact exploration types in INK1 compared to INK2 and INK3. According to Agerer (2001), ectomycorrhizal fungi at dry sites tend to have long-distance exploration types, while wet sites are dominated by contact types. Thus, the predominance of contact ectomycorrhizal fungi in INK1 is likely linked to the soil's higher water saturation potential, as indicated by its higher KS.

Additionally, the presence of the root pathogen might have influenced the ectomycorrhizal fungal community, favoring contact and medium-distance exploration types over long-distance types. The predominance of medium-distance ectomycorrhizal fungi in INK1 could be linked to the nitrogen uptake and nutritional needs of the chestnut trees, as previous studies indicate that contact exploration types are more efficient at acquiring labile nitrogen (Defrenne et al., 2019; Hobbie and Agerer, 2010). The reduced nitrogen availability in INK1 for the infected chestnut tree may also drive a shift in ectomycorrhizal preferences towards nitrogen-seeking fungi that explore greater distances compared to the contact and short-distance types found in INK2 and INK3.

5 Conclusion

This work highlights how the pedogenetic processes that shape the evolution of hillside soils can influence soil properties that promote the inoculation of ink disease pathogens in chestnut ecosystems of the Apennines. The disease condition creates instability within the soil-plant-microorganism system, which manifests not only as shifts in population dynamics within the rhizospheric soil but also in the regulation of nutrient cycles. Specifically, in the nitrogen cycle, it is possible that a nutritional deficiency for the plant arises due to an increased nitrogen immobilization process by rhizospheric microorganisms. These microorganisms find an abundance of labile carbon as a response to the stress of the diseased plant, but this is not the case for nitrogen, which is removed from available forms in the soil and immobilized in microbial biomass. Soil and soil-plant systems host complex microbial ecosystems and habitats, shaped by environmental conditions such as soil-forming factors and development processes. These environmental pressures select microbial communities with significant functional redundancy, i.e. while the specific taxa may vary, their functions remain stable. In other words, the microbial ecosystem is the result of an equilibrium among individual components, optimized for colonizing a habitat. Cooperation among microbial cells, transcending species, genus, or strain, creates the conditions that maximize resource use

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for microbial survival. From this perspective, it is often challenging to distinguish microbial metabolic pathways, processes, and their specific responses in soils.

A holistic approach to these problems is recommended, taking into account the genetic aspects of soil (horizon formation and morphological characteristics), which are shaped by various pedogenesis factors. The habitats and ecological niches of microbial communities, including pathogenic communities, can be influenced by soil properties and the diverse factors driving soil formation.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author contribution

Conceptualization: Livia Vittori Antisari; Data curation: William Trenti, Livia Vittori Antisari; Formal analysis: William Trenti, Federico Puliga, Livia Vittori Antisari; Funding acquisition: Livia Vittori Antisari; Investigation: William Trenti, Mauro De Feudis, Federico Puliga, Rosita Marabottini; Supervision: Alessandra Zambonelli, Fausto Gardini, Livia Vittori Antisari; Writing – original draft preparation: Mauro De Feudis, Federico Puliga; Writing – review & editing: Mauro De Feudis, Fausto Gardini, Sara Marinari, Sergio Murolo, Livia Vittori Antisari.

Competing interests

The authors declare that they have no competing interests.

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Appendix A

Sed *Soil physicochemical analyses*

The cation exchange capacity (CEC) and the exchangeable cations contents were determined according to the method proposed by Orsini and Rèmy (1976) and modified by Ciesielski and Sterckeman (1997) using 0.017 M hexamminecobalt(III)chloride as extracting solution and the amounts of Co and exchangeable cations were measured by inductively coupled plasma optical emission spectrometer (ICP–OES, Ametek, Spectro Arcos, Germany).

Microbial biomass C and N were estimated by the fumigation–extraction method using 0.5 M K_2SO_4 as extracting solution. Specifically, for each sample 10 g of 2–mm air dried soil was adjusted to 50% of field capacity and pre–incubated for 5 days. The soil samples were fumigated with $CHCl_3$ for 24 h at 25 °C. After, the fumigated and non−fumigated samples were mixed with 40 mL 0.5 M K_2SO_4 for 30 min on a horizontal shaker. The suspensions were filtered through 0.45µm membrane filter and C and N contents in the filtered solution were determined by a TOC–V CPN total organic carbon analyzer (Shimadzu, Japan). The microbial biomass C was calculated as EC/k_{EC}, where EC = (organic C extracted from fumigated soils) − (organic C extracted from non-fumigated soils) and k_{EC} = 0.45. Microbial biomass N was calculated as EN/k_{EN}, where EN = (total N extracted from fumigated soils) − (total N extracted from non-fumigated soils) and k_{EN} = 0.54. C and N inside the filtered solution obtained from non–fumigated soil samples were considered as water–extractable organic C (WEOC) and water– extractable N (WEN).

Basal respiration was determined by quantifying the CO_2 released in the process of microbial respiration during 28 days of incubation at 25°C of incubation after conditioning of the samples at 50% of their field capacity and a pre–incubation of 5 days. In particular, 10 g of 2–mm air dried soil sample was placed in 0.5 L jars with hermetic lids and after 1–3–7–10–14–21–28 days the beginning of incubation, the amount of CO_2 emitted from incubated soils was measured by alkali (1 M NaOH solution) absorption of the evolved CO_2 and titration of the residual OH– with a standardized HCl solution. The soil basal respiration (BR) of each soil sample was calculated as the average of the values measured during the incubation period.

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Appendix B

Table B1. Physicochemical characteristics of the soil under ill chestnut plants (INK1) and healthy chestnut plants (INK2 and INK3). CEC: cation exchange capacity; Caexch: exchangeable Ca; BS: base saturation.

Horizon		pH	Texture			CEC	Caexch	BS
			Sand	Silt	Clay			
			g kg^{-1}			$cmol_{(+)}kg^{-1}$		%
INK1								
A	2-10/12	5.78	203	584	213	20.36	8.23	49.22
AB	10/12-24	5.52	207	564	229	8.04	1.72	29.61
Bw(t)	24-30	5.70	266	475	259	8.76	2.01	31.31
BC	30-50+	5.50	237	562	201	10.59	2.46	30.74
INK2								
A1	1-13	6.42	202	675	123	19.45	7.18	43.86
A2	13-28	6.77	216	642	142	13.85	5.28	45.49
Bw(t)	28-40+	6.20	263	540	197	9.47	4.14	53.18
INK3								
A	0-5/9	6.40	267	618	115	14.46	6.53	53.77
AB	5/9-22	6.33	222	540	238	7.84	3.17	49.19
Bw(t)	22-40	6.61	248	539	213	8.86	3.71	50.12
BC1	40-49	6.76	205	618	177	9.67	4.41	53.56
BC2	49-60+	6.95	253	609	138	10.08	4.66	53.26

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Table B2. Hydraulic properties of the soil under ill chestnut plants (INK1) and healthy chestnut plants (INK2 and INK3). THS: water content at saturation; FC: water content at field capacity = -33 kPa; WP: water content at permanent wilting point = -1,500 kPa; AWC: available water capacity; KS: saturated hydraulic conductivity.

Horizon		THS	FC	WP	AWC	KS
		%	%	%	%	mm h-1
INK1						
A	2-10/12	68.5	58.7	20.3	38.5	10.3
AB	10/12-24	45.3	26.9	13.3	13.6	22.96
Bw(t)	24-30	49.6	31.1	18.7	12.4	31.08
BC	30-50+	44.0	27.8	12.3	15.5	23.94
INK2						
A1	1-13	59.9	46.5	11.6	34.8	0.28
A2	13-28	47.7	27.9	11.8	16.1	0.74
Bw(t)	28-40+	41.2	25.5	11.7	13.8	12.80
INK3						
A	0-5/9	49.0	29.2	9.9	19.3	1.07
AB	5/9-22	17.2	10.7	5.0	5.7	2.30
Bw(t)	22-40	44.1	28.4	12.7	15.7	16.55
BC1	40-49	33.4	20.0	8.8	11.1	10.11
BC2	49-60+	16.8	10.0	3.9	6.1	2.34

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Table B3. Values of properties related to organic matter in the soil under ill chestnut plants (INK1) and healthy chestnut plants (INK2 and INK3). OC: soil organic carbon; TN: total nitrogen; δ^{13} C: carbon isotopic ratio; δ^{15}N: nitrogen isotopic ratio; WEOC: water-extractable organic carbon; WEN: water-extractable nitrogen.

Horizon	OC	N	C:N	Cmic	SBR	WEOC	WEN	WEOC:OC	WEOC:WEN
	g kg^{-1}	g kg^{-1}		mg kg^{-1}	mg C-CO_2 kg s.s. $^{-1}$ h^{-1}	mg kg^{-1}	mg kg^{-1}	%	
INK1									
A	87.5	5.64	15.5	500.00	7.00	540	60.0	0.62	9.00
AB	18.7	1.50	12.5	20.40	1.57	481	26.4	2.57	18.23
Bw(t)	12.0	1.04	11.5	10.00	1.19	318	21.2	2.66	15.00
BC	12.5	1.08	11.5	44.00	1.22	266	15.2	2.14	17.52
INK2									
A1	52.12	3.90	13.4	400.00	4.16	290	60.0	0.56	4.83
A2	29.23	2.65	11.0	204.56	2.08	180	31.0	0.62	5.81
Bw(t)	13.69	1.46	9.4	32.24	1.15	165	20.4	1.20	8.07
INK3									
A	50.9	3.43	14.9	430.04	5.35	260	36.5	0.51	7.14
AB	13.5	1.24	10.8	86.08	1.38	166	19.6	1.23	8.47
Bw(t)	9.3	1.07	8.7	27.44	1.45	158	15.2	1.69	10.39
BC1	9.0	1.02	8.9	10.00	1.60	163	14.9	1.81	10.93
BC2	9.8	1.11	8.9	25.80	1.84	148	13.3	1.51	11.18

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Table B4. Enzimatic activities in the soil under ill chestnut plants (INK1) and healthy chestnut plants (INK2 and INK3). CELL (cellulase), NAG (chitinase), BG (β-glucosidase), AG (α-glucosidase), PHOS (phosphatase), SU (sulpha tase), XY (xylase), BUT (butyrase), LAP (leucine aminopeptidase), SEI (Synthetic Enzymes Index); OC (organic C); Cmic (microbial biomass-C).

Horizon	CELL nmol MUF h^{-1}	NAG mg C^{-1}	BG	AG	PHOS	SU	XY	BUT	LAP	SEI	SEI/OC	SEI/Cmic
INK 1												
A	73.2	303.2	272.4	27.3	640.7	296.8	62.6	571.7	797.7	3045	35	12
AB	3.5	17.4	11.0	2.9	183.5	69.3	6.7	223.4	116.1	634	31	62
Bw(t)	2.5	11.9	8.6	2.9	83.4	32.8	6.7	120.8	87.1	357	99	71
BC	0.7	3.7	4.9	1.9	36.1	22.7	2.7	71.4	70.6	215	17	10
INK 2												
A1	35.8	88.0	115.4	11.9	347.8	161.4	35.7	349.2	596.0	1741	33	9
A2	5.3	29.4	32.5	4.3	118.6	74.2	9.0	162.0	348.6	784	27	8
ABw(t)	30.1	24.1	13.2	3.0	100.2	33.1	5.3	108.7	95.0	413	30	25
INK 3												
A	26.7	68.9	141.5	11.0	228.5	88.2	22.9	278.5	489.6	1356	27	6
ABt	1.9	8.0	10.6	2.8	78.0	31.2	3.6	113.0	88.8	338	25	8
Bw(t)	1.6	4.1	6.3	1.0	35.7	18.5	2.7	66.8	66.5	203	22	15
BC1	1.1	1.8	2.6	0.2	22.3	10.3	0.5	37.0	121.5	197	22	39
BC2	2.2	5.7	4.5	3.9	25.2	12.4	2.6	46.1	73.8	176	18	14

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Appendix C

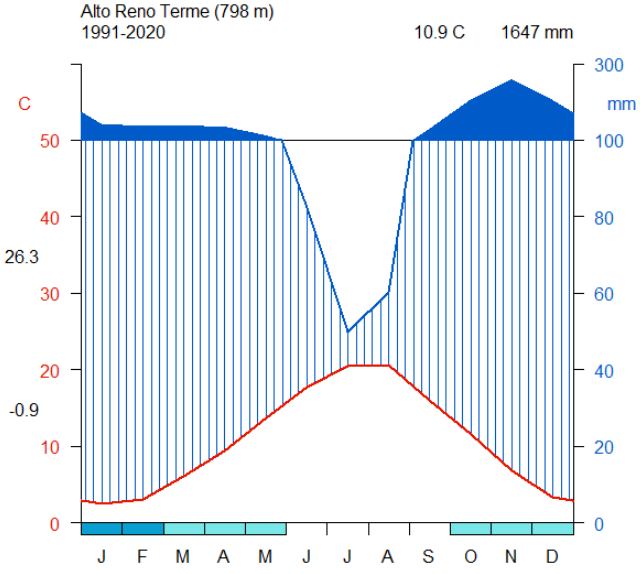

Figure C1. Walter & Lieth climatic diagram of the municipality of Alto Reno Terme for the period 1991-2020. Numbers at the top indicate mean annual temperature and annual precipitation; numbers on the left indicate daily maximum average temperature of the hottest month and daily minimum average temperature of the coldest month. Plotted with R package "climatol".

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Figure C2. Pictures showing symptomatic chestnut plant 1 m uphill from INK1 soil profile (a), and asymptomatic chestnut plants close to INK2 (b) and INK3 (c) soil profiles.

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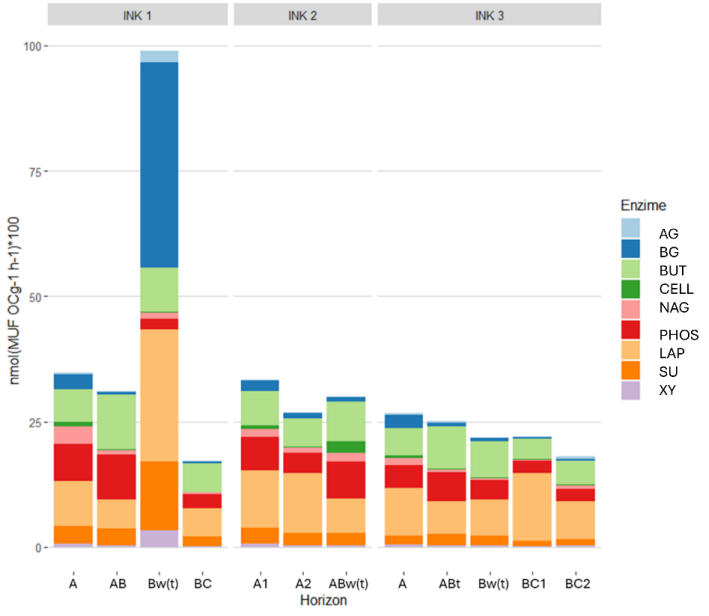
50 **(a)**



(b)

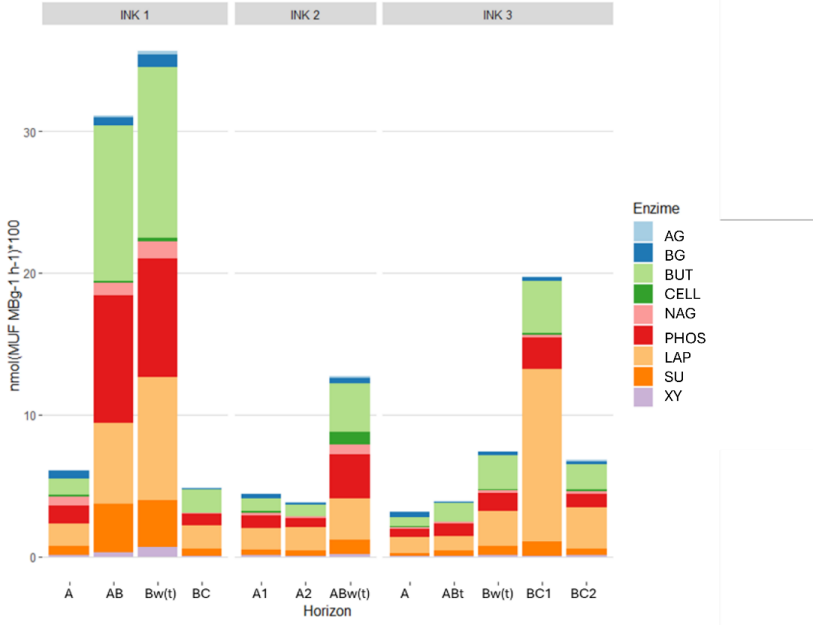


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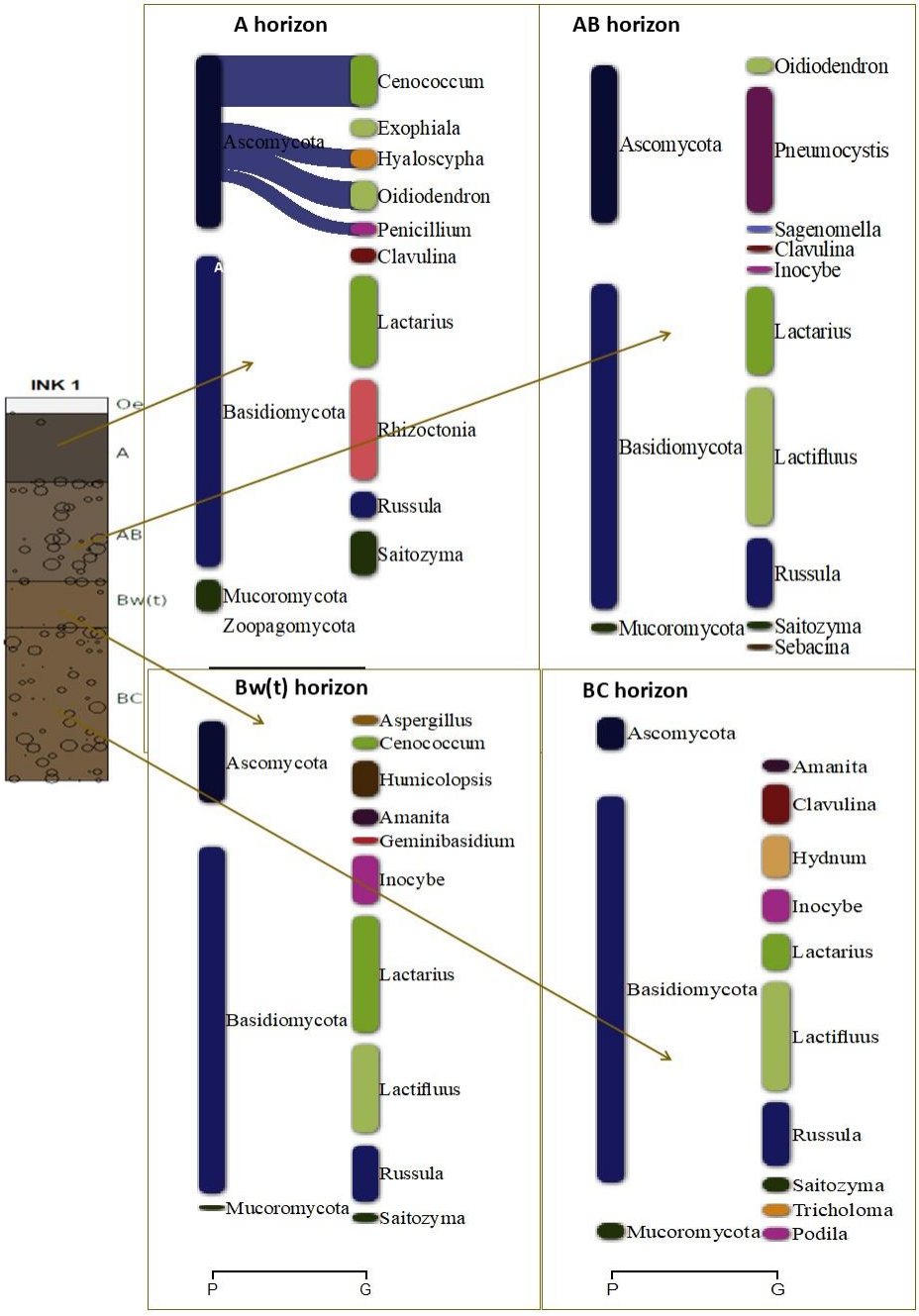
Figure C3. Specific enzymatic activities along the horizons of soil profiles per unit of OC (a) and Cmic (b). CELL (cellulase), NAG (chitinase), BG (β-glucosidase), AG (α-glucosidase), PHOS (phosphatase), SU (sulpha tase), XY (xylase), BUT (butyrase), LAP (leucine aminopeptidase).



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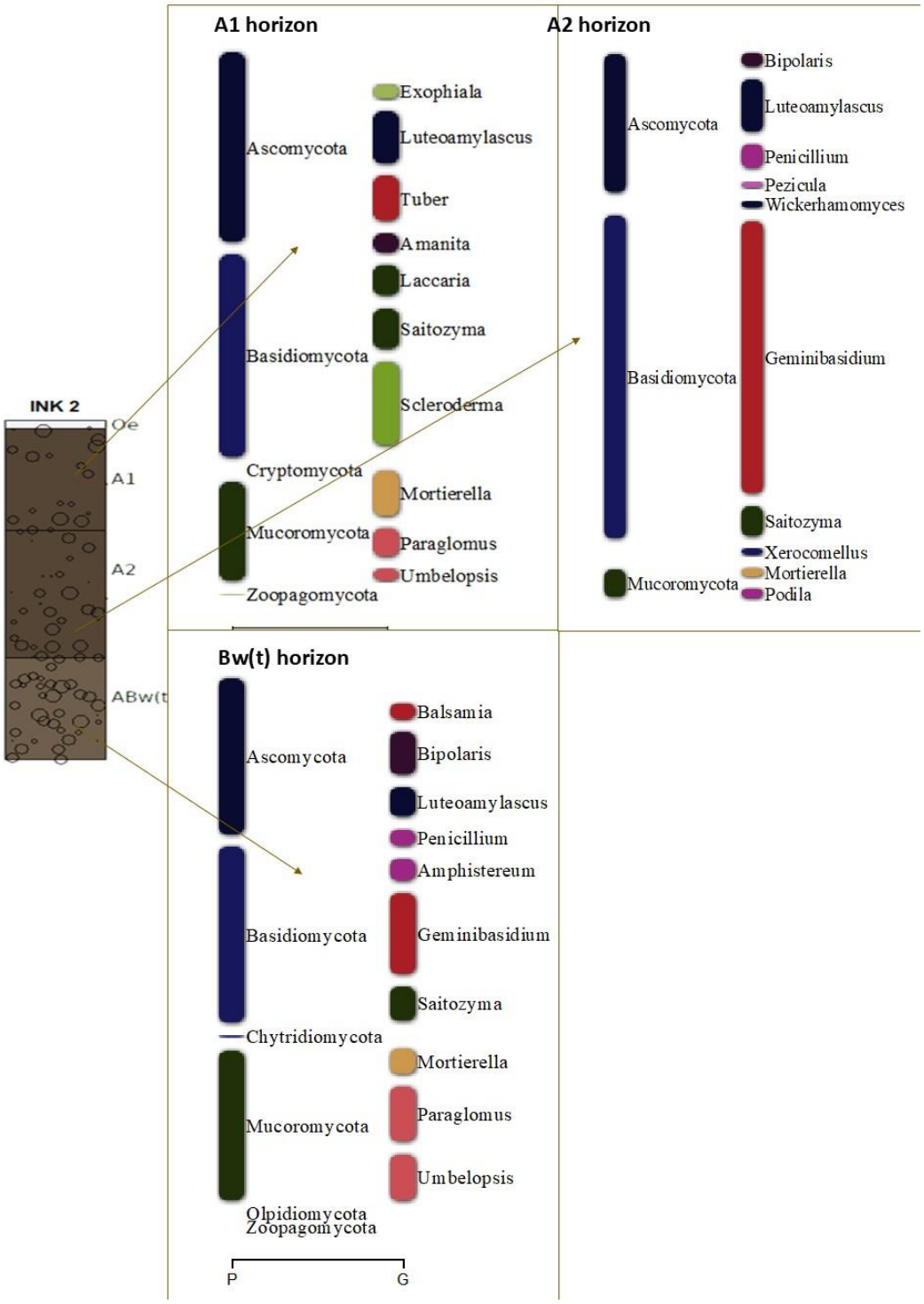

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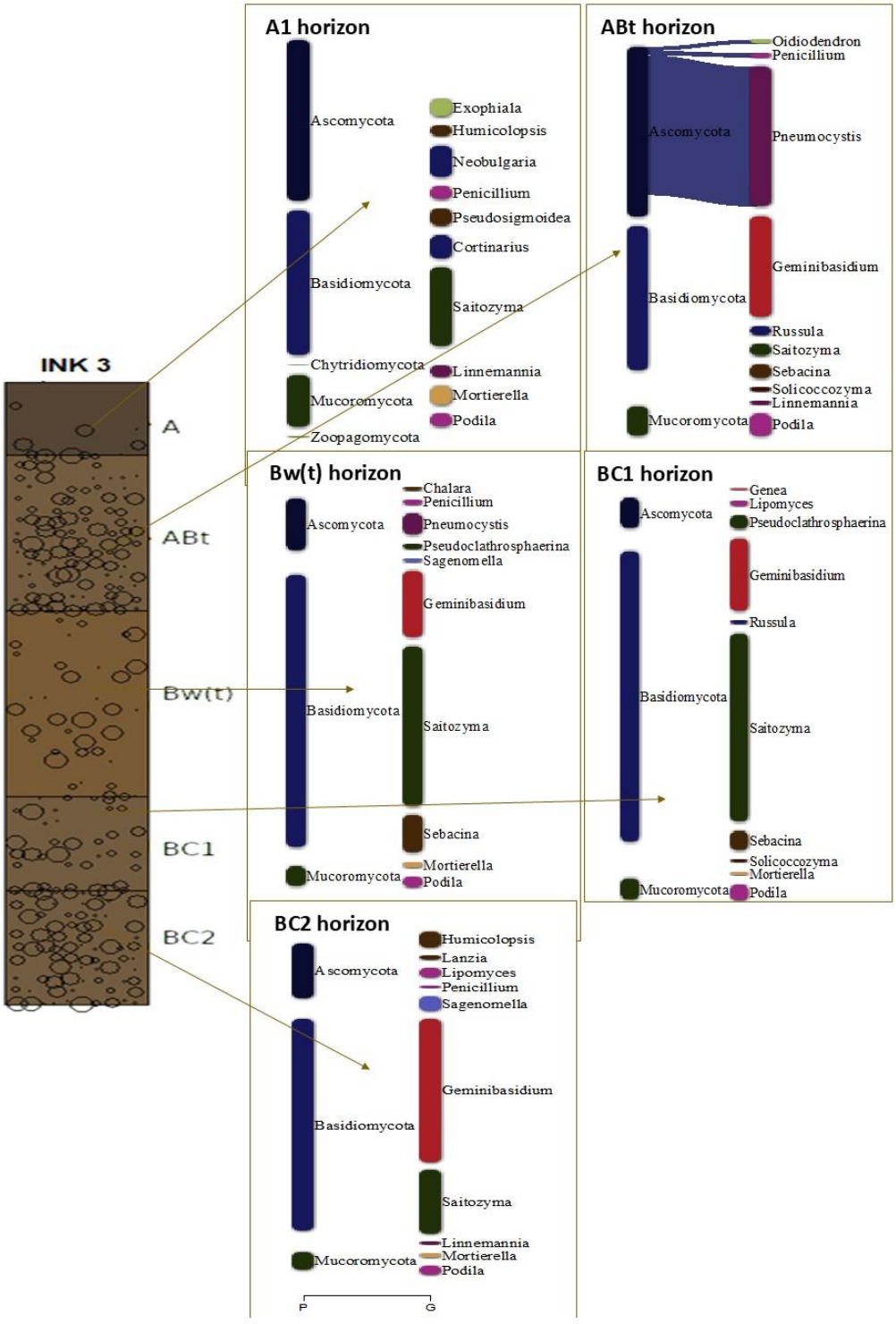

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Figure C4. Sankey diagram of the 10 most representative genera for each soil horizon and site: a) INK1 profile, b) INK2 profile and c) INK3 profile.