

Tree age progressively shapes grapevine rhizosphere microbial activity and nitrogen nutrition in a temperate vitiforestry system

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Research Article

Keywords: Agroforestry, grapevines, microbial enzymatic activity, nitrogen status, soil

Posted Date: July 14th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10142440/v1>

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Version of Record: A version of this preprint was published at Plant and Soil on October 9th, 2026. See the published version at <https://doi.org/10.1007/s11104-026-09166-3>.

Abstract

Background and Aims

Agroforestry, i.e., the planting of trees within agricultural systems, has been shown to enhance soil fertility, biodiversity, and the functioning of ecosystems. In temperate vineyards, vitiforestry represents a new type of agroforestry combining perennial grapevines and trees, either within or around vine rows. However, the effects of trees planted within vine rows on belowground microbial processes and grapevine growth remain underexplored.

Methods

In this study, we investigate soil physicochemical properties, microbial enzymatic activities associated with carbon (C), nitrogen (N), and phosphorus (P) acquisition, and nutrient acquisition strategies in bulk soil and grapevine rhizosphere compartments in a vineyard where trees were planted within vine rows either five years ago (5YO) or ten years ago (10YO).

Results

The results indicated that soil organic C and total N contents were higher near 10YO trees compared with soils from control rows (i.e., far from trees). Enzymatic activities increased in the rhizosphere of grapevines planted near trees compared with those planted farther away, and this effect was more pronounced near 10YO trees. Vector analysis revealed a shift toward greater relative investment in P-acquiring enzymes near older trees. The presence of trees also had a positive influence on vine nitrogen status without affecting berry yield per vine.

Conclusion

Overall, trees enhanced grapevine rhizosphere enzymatic activities, promoted nitrogen acquisition, and improved soil quality, offering a practical pathway toward more resilient and sustainable viticultural systems.

Introduction

The agricultural sector is currently facing numerous challenges, including adaptation to climate change, new requirements to reduce agricultural inputs, and the decline in certain crops. Addressing these issues requires a profound rethinking of cropping systems toward practices that are more sustainable, resilient, resource-efficient, and ecologically grounded. Agroecological practices have emerged as key strategies to mitigate climate-related stresses while maintaining soil fertility, biodiversity, and long-term productivity. Defined as the intentional planting of trees alongside crops and/or livestock within the same land-use system, agroforestry constitutes a cornerstone of the agroecological transition. Practiced worldwide, it encompasses a wide range of systems, from traditional multistrata arrangements common in tropical regions (Nair et al., 2017) to modern temperate practices such as alley-cropping and

silvopastoral systems (Amorim et al., 2023). Agroforestry provides multiple environmental and agronomic benefits, including improved microclimate regulation (Niether et al., 2018), enhanced soil structure and fertility, and increased soil C sequestration (Cardinael et al., 2018; Pan et al., 2025). It is increasingly recognized as an effective approach to enhance ecosystem functioning, soil fertility, and biodiversity while maintaining or even improving crop productivity (Cardinael et al., 2017; Lovell et al., 2018). Trees influence soil properties through numerous processes (Rhoades, 1997), and tree-soil-crop interactions have been investigated for decades (Szott et al., 1991; Lehmann et al., 1998; Schroth, 1998; Favor and Udawatta, 2021). In agroforestry systems, C inputs are generally higher than in treeless croplands (Jose, 2009; Cardinael et al., 2018) due to tree litterfall (Negash and Starr, 2021), pruning residues (Schneiderwind et al., 2019), and root-derived inputs (Germon et al., 2016). However, despite the well-documented benefits of agroforestry on soil functioning and ecosystem services, the mechanisms through which trees influence belowground microbial processes and nutrient dynamics in perennial cropping systems remain insufficiently understood, particularly in vineyard agroforestry systems.

Viticulture, like many agricultural sectors, is confronted with major environmental challenges, including biodiversity loss, declining ecosystem services, and climate change (Winkler et al., 2017; Giffard et al., 2022; Rusch, 2025), which may compromise the long-term sustainability and quality of wine production (van Leeuwen and Darriet, 2016; Candiago et al., 2023). Although trees have historically been present in vineyards, modern viticulture rarely employs agroforestry practices, partly due to the perceived risks of above- and below-ground competition between trees and vines (Grimaldi, 2018; Bourgade et al., 2020; Favor et al., 2024). With the exception of some vineyards in Argentina, Portugal, Spain, Nepal, Italy, Iran, and Greece, vitiforestry remains uncommon (Favor and Udawatta, 2021). In general, vitiforestry involves the association of two or more perennial plant species, leading to complex and long-lasting interactions among coexisting partners that share soil and atmospheric resources over extended periods. These interactions are expected to strongly influence belowground ecological processes, including microbial dynamics (Beule et al., 2022), nutrient cycling, and root-soil feedbacks (Bardgett and van der Putten, 2014). While the positive effects of agroforestry on aboveground biodiversity and ecosystem services are now well-established (Tamburini et al., 2020; Beillouin et al., 2021), increasing attention has recently been directed toward belowground biodiversity and soil microbiomes, recognized as key drivers of ecosystem functioning, nutrient cycling and plant health (Bakker et al., 2019). However, the way in which plant-associated microbial communities respond to agroforestry systems remains comparatively understudied. In vitiforestry specifically, only a limited number of studies have explored the diversity and composition of microbial communities and their associated biological activity (Lehr et al., 2025; Durney et al., 2026).

Soil microorganisms play a pivotal role in nutrient cycling, organic matter turnover, and the maintenance of soil fertility (van der Heijden et al., 2008). As such, the soil microbiome, defined as the community of microorganisms and their functional environment (Berg et al., 2020), constitutes a key biological indicator of plant health and productivity (Trivedi et al., 2020). By driving biogeochemical cycles, microorganisms regulate soil quality and ecosystem functioning through the transformation and mineralization of organic matter, thereby controlling the availability of essential nutrients such as C, N,

and P (Schimel and Schaeffer, 2012). In particular, the rhizosphere, defined as the narrow soil zone surrounding plant roots, represents a highly biologically active compartment where root exudates shape microbial community composition and activity (Philippot et al., 2013; Reinhold-Hurek et al., 2015; Banerjee et al., 2016). These microorganisms contribute to plant nutrition and soil functioning through nutrient cycling, P solubilization, and the production of extracellular enzymes involved in organic matter decomposition (Fanin et al., 2021). Extracellular enzyme activities (EEA) reflect the potential capacity of soil microbial communities to acquire C, N, and P from complex organic substrates (Sinsabaugh et al., 2008; Fanin et al., 2022), and therefore provide valuable insights into microbial functioning and soil biogeochemical processes (Nannipieri et al., 2012; Burns et al., 2013; Bradford et al., 2021). Since microbial decomposer communities process substrates with varying C:N:P stoichiometries relative to their own biomass (Cleveland and Liptzin, 2007; Mooshammer et al., 2014), changes in the relative abundance of C-, N-, and P-acquiring enzymes may reveal shifts in microbial nutrient acquisition strategies and nutrient limitation (Sinsabaugh et al., 2009; Moorhead et al., 2016). Understanding how the presence of trees influences these microbial processes therefore appears essential for evaluating the impact of vitiforestry on soil functioning and grapevine nutrition. However, despite growing interest in vineyard agroforestry systems, the impact of the age and presence of trees on microbial functioning and nutrient acquisition strategies in both bulk soil and grapevine rhizosphere compartments remains widely undocumented.

Trees can strongly influence soil physicochemical and biological properties through litter inputs, root exudation, microclimate and moisture regulation, and nutrient redistribution via deep rooting (Maeght et al., 2013; Bardgett and van der Putten, 2014; Cardinael et al., 2017). Through these processes, trees are expected to shape microbial activity and nutrient cycling within agroforestry systems. As trees grow, these effects may gradually intensify, reshaping the soil-microbe-root interface over time, suggesting that tree age could act as a major driver of belowground functional dynamics. In agroforestry systems, significant increases in soil organic carbon (SOC), defined as the C stored in soil organic matter, generally emerge only after long-term establishment, typically beyond 15 years and up to several decades (Oelbermann et al., 2006; Gupta et al., 2009; Mao et al., 2012; Tian et al., 2013; Pardon et al., 2017). However, such changes tend to remain weak or non-detectable during the first years following tree establishment, particularly in systems younger than five years (Clivot et al., 2020). These findings highlight the importance of considering temporal dynamics when evaluating the impact of agroforestry on soil functioning. However, despite growing interest in vitiforestry systems, the role of tree age in shaping soil microbial functioning and enzymatic activities in both rhizosphere and bulk soil compartments remains largely undocumented. Understanding these temporal dynamics is therefore essential when it comes to assessing the long-term effects of vitiforestry on soil functioning and grapevine nutrition.

In this study, our main objective was to assess the effect of tree age, expressed as the number of years since planting, on soil physicochemical properties, microbial functioning, and grapevine nutritional status in a vineyard under real production conditions. Since the vines were cultivated under the same pedoclimatic context and shared an identical vineyard management history, this design allowed us to

assess the localized effects of trees while minimizing confounding factors related to site conditions or management practices. We investigated how the presence of trees influenced soil composition and microbial functioning in both bulk soil and grapevine rhizosphere compartments in a 16-year-old vineyard plot established in 2008. Sampling was conducted in spring 2024 in vine rows where plum trees and field maple trees were planted in 2014 (i.e., 10-year-old trees at the time of sampling, hereafter 10YO), and in rows where plum trees and quince trees were planted in 2019 (5-year-old trees, 5YO). Soil and rhizosphere samples were also collected from vine rows located farthest from the trees, which were used as the control condition (i.e., vine rows without trees nearby) (Fig. 1). Four groups of indicators were assessed: (i) soil physicochemical properties, (ii) enzymatic activities involved in C, N, and P cycling in bulk soil and the grapevine rhizosphere, (iii) the stoichiometric balance of microbial nutrient acquisition strategies, and (iv) grapevine physiological parameters measured throughout the 2024 growing season to assess plant nutritional status, yield, and berry quality. We hypothesized that (i) the presence of trees would enhance soil organic matter content and microbial enzymatic activities, (ii) these effects would become stronger as the age of the trees increased, reflecting the gradual establishment of tree-soil-microbe interactions over time, and (iii) improved soil microbial functioning near trees would positively influence grapevine nutritional status without negatively impacting yields. To assess the effect of the duration of tree-vine co-existence, samples from vines associated with plum trees (5YO and 10YO) were compared with those from control vines.

Material and Methods

Study site

The study was conducted in an organic vineyard located in Lapouyade, within the Bordeaux appellation (France; 45°05'12.6"N, 0°17'33.6"W). The site is characterized by an oceanic climate and sandy soil overlying a moderately clayey subsoil. The vineyard was established in 2008 with *Vitis vinifera* cv. Merlot grafted onto 101 – 14 MGt rootstock, at a planting density of 6,600 vines ha⁻¹ and an inter-row spacing of 1.5 m. Prior to vineyard establishment, the land was used for grazing. The plot is bordered by a road, a forest, and neighboring vineyards, and presents a mean slope of approximately 4%. Vineyard management is organic, without manure application, and includes permanent grass cover combined with mechanical cultivation beneath vine rows. Vines are trained using a short-cordon (*cordon de Royat*) pruning system, and mowing frequency is voluntarily reduced to promote biodiversity. Since vineyard establishment, several tree-planting campaigns have been conducted within vine rows.

The present study focused on vine rows where trees were planted in 2014 and 2019. Tree spacing within rows ranged from 5 to 9 m, and several fruit and forest tree species were introduced. Three tree species were selected for this study: plum trees (*Prunus domestica* L.), quince trees (*Cydonia oblonga* Mill.), and field maples (*Acer campestre* L.). They included plum trees and quince trees planted in 2019 (5YO) and plum trees and field maples planted in 2014 (10YO). Six trees per species and planting year were selected, together with twelve grapevines located in distant rows without trees nearby, used as the

control condition. The experimental design and the positions of sampled trees and grapevines are shown in Fig. 1.

Soil and rhizosphere sampling

Soil and rhizosphere samples were collected in spring 2024 (23 April) for enzymatic analyses, while additional bulk soil samples for physicochemical analyses were collected on 16 July 2024. All samples were collected from the 0–30 cm soil layer using a hand auger at predefined sampling locations. Bulk soil was sampled approximately 50 cm from the tree trunk, both within the vine row and in the adjacent inter-row. In control areas, soil was collected following the same sampling pattern at 50 cm from the grapevine trunk. Samples were placed in plastic bags, transported in an icebox, and returned to the laboratory on the same day. They were stored overnight in a cold room and processed the following day. Soil samples were sieved through a 2 mm stainless-steel sieve. For enzymatic analyses, subsamples were stored at -80°C , and a fraction of each sample was lyophilized and weighed before and after freeze-drying to determine the soil moisture content.

For each tree, the rhizosphere of two nearby grapevines was sampled: one grapevine planted in the same row as the tree and one planted in the adjacent row (Fig. 1). In total, 12 grapevine rhizosphere samples were collected for each tree species and planting year, together with 12 rhizosphere samples from control grapevines. The roots were carefully excavated and transferred into plastic bags together with the adhering soil. In the laboratory, soil tightly attached to the roots was gently detached by hand, sieved, and transferred into sterile tubes. As for bulk soil, a subsample was lyophilized to determine the moisture content, whereas the remaining rhizosphere material was stored at -80°C until further analysis. For enzymatic assays, composite samples were prepared by pooling equal proportions of rhizosphere soil collected from the two grapevines associated with each tree, or from two control grapevines. Consequently, the final number of independent samples used for rhizosphere enzymatic analysis was $n = 6$ per condition.

Soil chemical analysis

Samples for soil physicochemical characterization were analyzed by an accredited laboratory (QUALYSE, France). Total C (SOC) was determined by dry combustion (NF ISO 10694) and expressed as soil organic matter (SOM, %) using the conventional conversion factor ($\text{SOM} = \text{SOC} \times 1.72$). Total nitrogen (TN, %; NF ISO 13878), C:N ratio (unitless), soil pH measured in water ($\text{pH}_{\text{H}_2\text{O}}$; NF EN ISO 10390) and in KCl (pH_{KCl} ; NF EN ISO 10390), exchangeable potassium (K_2O , mg kg^{-1} ; NF X 31–108), exchangeable magnesium (MgO , mg kg^{-1} ; NF X 31–108), exchangeable manganese (Mn, mg kg^{-1} ; NF X 31–120), and total sulfate (SO_4^{2-} , mg kg^{-1} , Scott method) were calculated. Extractable P was determined using the Olsen method (P_{Olsen} , mg kg^{-1} ; NF ISO 11263) and the Dyer method (P_{Dyer} , mg kg^{-1} ; NF X 31–160). Elemental concentrations of potassium, magnesium and P were calculated from oxide equivalents (K_2O ,

MgO and P₂O₅) using standard stoichiometric conversion factors derived from molecular weights ($K = K_2O \times 0.83$; $Mg = MgO \times 0.60$; $P = P_2O_5 \times 0.436$).

Enzymatic activities

The potential activities of seven hydrolytic soil enzymes involved in the degradation of organic C, N, and P were measured following the protocol of Bell et al. (2013) with slight modifications. The selected enzymes were associated with the acquisition of the three major nutrients. C acquisition was represented by the activities of β -1,4-glucosidase (BG, EC 3.2.1.21), β -D-cellobiosidase (CBH, cellobiohydrolase; EC 3.2.1.91), α -1,4-glucosidase (AG, EC 3.2.1.20), and β -xylosidase (XYL, EC 3.2.1.37). N acquisition was represented by β -1,4-N-acetylglucosaminidase (NAG, EC 3.2.1.52), and L-leucine aminopeptidase (LAP, EC 3.4.11.1). Meanwhile, P acquisition was represented by phosphatase (AP, EC 3.1.3.2). Enzyme activities were determined using fluorogenic substrates containing either 4-methylumbelliferone (4-MUB) for six of the enzymes or 7-amino-4-methylcoumarin (7-AMC) for L-leucine aminopeptidase. For each replicate, 2.75 g of soil was homogenized in 91 mL of 0.1 M Tris–HCl extraction buffer adjusted to a pH of 7.0. The suspension was blended for one minute to obtain a uniform slurry. Aliquots of 800 μ L of this homogenate were dispensed into the wells of 96-deep-well microplates containing 200 μ L of each substrate at a final concentration of 200 μ M. Substrate concentrations were provided in excess to measure potential enzymatic activities. Standard curves of 4-MUB and 7-AMC (0–100 μ M) were prepared in parallel using both buffer and soil slurries to account for quenching effects. Microplates were incubated for three hours at 25°C in the dark, with gentle agitation every 30 minutes to maintain suspension. Following incubation, the plates were centrifuged at 3,000 rpm for three minutes, and 250 μ L of the supernatant from each well was transferred to black flat-bottom 96-well reading plates. Fluorescence was quantified using a Synergy H1M Biotek® microplate reader with excitation at 365 nm and emission at 450 nm. Enzyme activities are expressed as nmol g⁻¹ h⁻¹ (DW), reflecting the potential rate of substrate degradation by the microbial community under standardized assay conditions. Prior to calculating the total C-, N-, and P-acquiring enzyme activities, individual enzyme activities were converted to mmol kg⁻¹ and totaled within each category.

Vector analysis of enzymatic stoichiometry was performed according to the framework proposed by Moorhead et al. (2016) and Fanin et al. (2016) to assess the relative allocation of microbial enzymatic investment toward C, N, and P acquisition. This approach derives two complementary metrics (vector length and vector angle) from the proportional relationships among C-, N-, and P-acquiring enzyme activities. Vector analyses were calculated using BG activity for C-acquiring enzymes, the sum of NAG and LAP activities for N acquisition, and AP activity for P acquisition. The vector length reflects the relative microbial investment in C acquisition compared with nutrient acquisition, with longer vectors indicating stronger relative C limitation. The vector angle reflects the balance between P and N acquisition, with angles lower than 45° indicating greater relative investment toward N acquisition and angles higher than 45° indicating greater relative investment toward P acquisition (Moorhead et al.,

2016). This framework provides an integrative indicator of microbial resource investment and nutrient acquisition strategies in soils.

DNA extraction and quantification

A portion of soil and rhizosphere from each subsample were freeze-dried for 120 h using a Christ Alpha® 1–4 lyophilizer (Bioblock Scientific) and stored at -20°C. Genomic DNA was isolated from 250 mg of lyophilized soil using the DNeasy Power Soil Pro Kit (Qiagen) as described in Lailheugue et al. (2024). Extracted DNA was quantified on a Qubit® 3.0 fluorometer (Thermo Fisher Scientific) using the Qubit™ dsDNA HS Assay Kit, and its quality was checked using a NanoDrop™ 2000/2000c spectrophotometer (Thermo Fisher Scientific). Total extracted DNA concentration was used as a proxy for microbial biomass in soil and rhizosphere samples, as previously proposed in soil microbial ecology studies (Fornasier et al., 2014).

Grapevine physiological measurements

The nitrogen (N) status of grapevines was assessed in May 2024 using a hand-held fluorescence sensor (Dualox®, Force-A, France). This device provides non-destructive estimations of leaf chlorophyll (Chl; Dualox index), anthocyanin, and flavonol contents (Flav; Dualox index), and calculates the Nitrogen Balance Index (NBI), corresponding to the chlorophyll-to-flavonol ratio. The NBI is widely used as an indicator of the instantaneous N status of grapevines.

Petioles were collected at mid-*véraison* (30 July 2024). For each tree, three petioles were sampled from each of the four surrounding grapevines and pooled to obtain one composite sample (Fig. 1). To reduce potential heterogeneity between cane and spur positions, petioles were collected exclusively from shoots emerging from the cane. Since the phenological stage and phytomer position influence leaf mineral composition (Harris et al., 2021), special care was taken to sample petioles from leaves at the same phenological stage and phytomer position across all vines. Samples were oven-dried at 50°C and sent to Waypoint Analytical (Richmond, VA, USA) for mineral analyses. Thirteen mineral elements were quantified, including macronutrients (N, P, K, S, Mg, Ca, and Na) and micronutrients (B, Zn, Mn, Fe, Cu, and Al). Elemental concentrations were determined using inductively coupled plasma optical emission spectrometry (ICP-OES MS 730-ES), except for total N, which was measured using a Leco FP-528 nitrogen determinator. Macronutrient concentrations are expressed as a percentage of dry weight (% DW, w/w), and micronutrient concentrations as parts per million (ppm DW).

At harvest time, (12 September 2024), all bunches from each grapevine were weighed to estimate the yield per vine. A subsample of 100 berries per grapevine was collected in the field, weighed on-site, and transported to the laboratory under refrigerated conditions. The berries were crushed and the must was extracted using a BagMixer® 400W (Interscience, France). The juice was filtered (BagFilter®, Interscience, France) and centrifuged at 20°C for 10 mins at 10,000 rpm. The recovered supernatant (minimum 12 mL) was analyzed by Fourier Transform Infrared Spectroscopy (FTIR) using a WineScan™

analyzer (FOSS, Nanterre, France), following the manufacturer's calibrated "Must" method. The following parameters were determined: yeast assimilable nitrogen (YAN, mg N L⁻¹), estimated as the sum of ammonium nitrogen (NH₄⁺-N, mg N L⁻¹) and amino nitrogen (mg N L⁻¹), sugar concentration (g L⁻¹), density (g mL⁻¹), potential alcohol (% vol), pH, gluconic acid (g L⁻¹), malic acid (g L⁻¹), tartaric acid (g L⁻¹), total acidity (g L⁻¹ H₂SO₄), and volatile acidity (g L⁻¹ H₂SO₄).

For C isotope analyses, 5 µL of must were pipetted into tin capsules and oven-dried. Capsules were sent to the SILVATECH facility (INRAE, Nancy, France) for isotopic analyses. The C isotope composition (δ¹³C) was determined using an elemental analyzer coupled via a ConFlo IV interface to a continuous-flow isotope ratio mass spectrometer (EA IsoLink CN IRMS System: Flash IRMS Elemental Analyzer, ConFlo IV Universal Interface, Delta V Advantage IRMS; Thermo Fisher Scientific, Bremen, Germany). Samples were combusted at 1,025°C under excess oxygen. The resulting CO₂ was separated by temperature-ramped gas chromatography and analyzed using thermal conductivity detection and isotope ratio mass spectrometry. Results are expressed in ‰ relative to the Vienna Pee Dee Belemnite (VPDB) standard. Measurement uncertainty was ± 0.3‰ (2σ) for complex samples.

Statistical analyses

All statistical analyses were performed using R version 4.3.2 (R Core Team, 2023). The data were examined for normality using the Shapiro-Wilk test and for homogeneity of variances using Levene's test prior to parametric analyses. Enzymatic activities, microbial biomass, and vector parameters were analyzed separately for bulk soil and rhizosphere samples. Within each compartment, the effect of tree age (control, 5YO, 10YO) was tested using a one-way analysis of variance (ANOVA) when assumptions were met, followed by Tukey's honestly significant difference (HSD) post hoc tests. When normality or homoscedasticity assumptions were not satisfied, non-parametric Kruskal–Wallis tests were applied. Pairwise comparisons were conducted using Dunn's tests with Benjamini–Hochberg correction for multiple testing. Relationships among grapevine nitrogen-related physiological variables were assessed using Spearman's rank correlation coefficients (ρ) and visualized using correlation matrices. All graphical representations were generated using the ggplot2 package (Wickham, 2016). Statistical significance was set at $P < 0.05$, and results are presented as a mean ± standard deviation (SD) unless otherwise stated.

Results

Soil physicochemical properties

The full set of physicochemical parameters for soil samples collected near 5YO and 10YO plum trees, as well as control grapevines, is presented in Table 1. Soil organic matter (SOM) ranged from 2.00 ± 0.45% in soils near control grapevines to 2.93 ± 0.65% near 10YO plum trees. Total nitrogen (TN) ranged from 1.01 ± 0.15‰ in soils near control grapevines to 1.40 ± 0.28‰ near 10YO plum trees. The C:N ratio was

approximately 12 and did not differ significantly among treatments. Significant differences among treatments were observed for total SOC, the derived SOM, and total nitrogen (TN), which were significantly higher in soils associated with 10YO plum trees than in soils near control grapevines. Intermediate values, not significantly different from the control, were observed near 5YO trees (Table 1).

Macronutrients related to K and P availability showed no significant differences among treatments. Both oxide-equivalent concentrations (K_2O , MgO , and P_2O_5 measured by Dyer and Olsen extractions) and the corresponding elemental concentrations (K, Mg, and P) remained statistically similar across the three conditions. Likewise, the K:Mg ratio did not differ significantly. The soil pH, measured both in H_2O and KCl, was not significantly affected by the presence or age of the trees. Similarly, manganese (Mn) concentrations did not differ significantly among treatments. Only sulfate (SO_4^{2-}) concentrations were significantly higher in soils associated with 10YO trees compared with control vines. Similar results were obtained when tree age was considered independently of tree species (i.e., when 10YO plum trees and field maples were compared with 5YO plum trees, quince trees, and control grapevines) (Table S1). Higher SOC, SOM, and TN values were consistently observed in soils associated with older trees.

Table 1

Physicochemical parameters of bulk soils sampled 50 cm from 5YO and 10YO plum trees and from control vines. Soil samples were collected at a depth of 0–30 cm. Means ($\pm$ SD) are indicated ($n = 6$). Different letters indicate significant differences among groups ($P < 0.05$).

Variable	10YO plum trees	5YO plum trees	Control vines
Total soil organic carbon, SOC (%)	1.71 $\pm$ 0.38 a	1.36 $\pm$ 0.18 ab	1.16 $\pm$ 0.26 b
Soil organic matter, SOM (%)	2.93 $\pm$ 0.65 a	2.33 $\pm$ 0.31 ab	2 $\pm$ 0.45 b
Total nitrogen, TN (‰)	1.4 $\pm$ 0.28 a	1.12 $\pm$ 0.12 ab	1.01 $\pm$ 0.15 b
C:N ratio	12.13 $\pm$ 0.58 a	12.08 $\pm$ 0.48 a	11.43 $\pm$ 1.07 a
pH_H2O	6.28 $\pm$ 0.25 a	6.35 $\pm$ 0.42 a	6.53 $\pm$ 0.36 a
pH_KCl	5.42 $\pm$ 0.2 a	5.45 $\pm$ 0.48 a	5.55 $\pm$ 0.27 a
Oxide-equivalents concentrations			
K ₂ O (mg kg ⁻¹)	92.83 $\pm$ 64.21 a	81.67 $\pm$ 47.5 a	95 $\pm$ 42.38 a
MgO (mg kg ⁻¹)	204.5 $\pm$ 139.91 a	143.5 $\pm$ 77.55 a	216.33 $\pm$ 201.67 a
P2O5_Dyer (mg kg ⁻¹)	230.83 $\pm$ 68.78 a	198.67 $\pm$ 40.02 a	185.33 $\pm$ 30.51 a
P2O5_Olsen (mg kg ⁻¹)	57 $\pm$ 9.84 a	51.83 $\pm$ 6.55 a	47.33 $\pm$ 3.2 a
Elemental concentrations			
K (mg kg ⁻¹)	77.05 $\pm$ 53.29 a	67.78 $\pm$ 39.42 a	78.85 $\pm$ 35.18 a
Mg (mg kg ⁻¹)	122.7 $\pm$ 83.95 a	86.1 $\pm$ 46.53 a	129.8 $\pm$ 121 a
P_Dyer (mg kg ⁻¹)	100.64 $\pm$ 29.99 a	86.62 $\pm$ 17.45 a	80.81 $\pm$ 13.3 a
P_Olsen (mg kg ⁻¹)	24.85 $\pm$ 4.29 a	22.6 $\pm$ 2.86 a	20.64 $\pm$ 1.4 a
K.Mg	0.64 $\pm$ 0.26 a	0.8 $\pm$ 0.28 a	0.82 $\pm$ 0.35 a
Other			
Mn (mg kg ⁻¹)	16.68 $\pm$ 7.53 a	18.2 $\pm$ 4.02 a	14.3 $\pm$ 5.43 a
SO ₄ ²⁻ (mg kg ⁻¹)	67.68 $\pm$ 38.36 a	43.22 $\pm$ 11.29 ab	35.55 $\pm$ 4.32 b

Enzymatic activities

Enzymatic activities displayed strong variations depending on the age of the tree and soil compartment (Fig. 2, Fig. 3, Fig. S1 and Fig. S2). In bulk soil (Fig. 2 and Fig. S1), the activities of C-acquiring enzymes (BG, CBH, XYL, and AG; Fig. 2A) did not differ significantly among soils collected near plum trees of different ages or control vines, although CBH activity was significantly higher in control soils compared

with soils near 5YO trees. Similarly, N-acquiring enzymes (NAG and LAP) displayed contrasted responses across treatments (Fig. 2B). Although NAG activity tended to decrease in soils associated with older trees, this trend was not supported by statistical analysis. In contrast, LAP activity was significantly higher in control soils compared with soils near 5YO trees, while intermediate values were observed near 10YO trees. The P-acquiring enzyme phosphatase (AP) tended to show the opposite pattern, with slightly higher activity near older trees, although differences among treatments were not statistically significant (Fig. 2C). In the vine rhizosphere, C-acquiring enzymes displayed a clearer pattern (Fig. 3 and Fig. S1). The activity of all C-related enzymes was significantly higher in the rhizosphere of grapevines near 10YO trees than in those near 5YO trees and control vines (Fig. 3A). N-acquiring enzymes followed a similar trend, reflecting enhanced protein degradation and N turnover in the rhizosphere of grapevines adjacent to older trees (Fig. 3B). For P acquisition, AP activity was significantly higher in the rhizosphere of grapevines near 10YO trees compared with that of grapevines near 5YO trees and control vines (Fig. 3C).

A similar pattern was observed in both bulk soil and grapevine rhizosphere compartments when the effect of tree age was evaluated across all tree species (Fig. S2), further supporting previous observations. Interestingly, the soil-to-rhizosphere enzymatic activity ratio (Fig. S3A) generally indicated higher activities in bulk soil than in the rhizosphere, although this pattern depended on the enzyme considered. Ratios were particularly high for several C-acquiring enzymes, whereas NAG consistently exhibited higher activity in the rhizosphere than in bulk soil. Overall, soil-to-rhizosphere activity ratios tended to decrease near older trees compared with control vines, suggesting a relative functional activation of the rhizosphere compartment in the vicinity of trees. The degree of functional coupling between bulk soil and rhizosphere enzyme activities was assessed using Spearman correlation coefficients (ρ ; Fig. S3B). Significant positive correlations indicated that variations in enzymatic activities were generally coordinated between the two compartments, suggesting that factors influencing enzyme production or stabilization may operate similarly in both environments.

Vector length did not differ significantly among treatments in bulk soil, indicating no detectable differences in relative microbial investment toward C acquisition across conditions. In the rhizosphere, vector length was significantly higher in grapevines located near 5YO trees than in control grapevines, whereas values near 10YO trees were intermediate and did not differ significantly from the other two groups. This pattern suggests an increased relative microbial investment in C acquisition in the rhizosphere of grapevines growing near younger trees. Vector angles were generally above 45° in bulk soil, indicating a tendency toward proportionally greater investment in P-acquiring enzymes relative to N-acquiring enzymes. In bulk soil, vector angles increased with tree age and were significantly higher under 10YO trees compared with the control treatment ($P < 0.05$), while values under 5YO trees were intermediate and did not differ significantly from the other two groups. This pattern suggests a shift toward greater microbial investment in P-acquiring enzymes in soils associated with older trees. In contrast, no significant differences in vector angle were detected among treatments in the rhizosphere, although vector angles tended to be higher in the presence of trees.

Microbial biomass

The microbial biomass, estimated based on the quantity of DNA extracted from samples, did not differ significantly among treatments in either bulk soil or grapevine rhizosphere compartments (Table 2), suggesting that total microbial abundance was not significantly affected by the presence or age of the trees.

Table 2
Microbial biomass estimated based on the DNA content ($\mu\text{g g}^{-1}$ soil) in bulk soil and in the grapevine rhizosphere under three conditions: grapevines associated with 10YO plum trees, 5YO plum trees, and control grapevines without trees. Means ($\pm$ SD) are indicated ($n = 6$). An identical letter indicates no significant differences among groups ($P < 0.05$).

Rhizosphere ($\mu\text{g g}^{-1}$ soil)	10YO plum trees	5YO plum trees	Control vines
	7.60 $\pm$ 1.09 a	6.55 $\pm$ 2.78 a	7.06 $\pm$ 2.67 a
Bulk soil ($\mu\text{g g}^{-1}$ soil)	8.35 $\pm$ 4.20 a	6.39 $\pm$ 4.06 a	7.04 $\pm$ 4.02 a

Grapevine physiological measurements

Physiological, phenotypic, and chemical measurements of grapevines are presented in Tables S2 and S3 and Fig. 5. Significant differences were observed for Dualex measurements performed in May during the vegetative growth phase (Table S2 and Fig. 5A). Grapevines growing in proximity to older trees exhibited higher NBI values than those planted near younger trees and control grapevines, indicating an improved nitrogen status (Tables S2 and S3). Interestingly, although other nitrogen-related variables, such as petiole N concentration or must nitrogen content, did not show statistically significant differences among treatments (Tables S2 and S3), strong positive correlations were observed between NBI and other indicators of grapevine nitrogen nutrition (Fig. 5B). In particular, the NBI value was positively correlated with petiole nitrogen concentration (% DW) and ammoniacal nitrogen concentration (mg N L^{-1}) in must.

Overall, no major differences in petiole macro- and micronutrient concentrations were observed among treatments (Tables S2 and S3). The only exception concerned copper concentrations, which were significantly lower near 10YO plum trees compared with the other conditions. The C isotope composition ($\delta^{13}\text{C}$) did not differ significantly among treatments. Although mean $\delta^{13}\text{C}$ values were slightly lower (approximately 1‰) in vines growing near trees compared with control grapevines, this difference was not statistically significant and should therefore be interpreted with caution. Under the conditions of this study, the proximity of the trees did not have a detectable impact on grapevine C isotope composition. No significant impacts were detected on yield components. Similarly, the majority of must composition parameters did not differ significantly among treatments. However, berry must from vines growing near older trees exhibited higher gluconic and tartaric acid contents than control vines, resulting in higher total acidity.

Multivariate relationships between enzymatic activities, soil properties, and grapevine physiological variables

The redundancy analysis revealed that the distribution of samples among treatments was mainly expressed along the second constrained axis (RDA2), which explained 31% of the variance (Fig. 6). Samples associated with 10YO plum trees were generally clustered in the lower part of the ordination space and were associated with several rhizosphere enzymatic activities, including BG, CBH, AP, and XYL, as well as with higher total SOC and total nitrogen (TN) contents. In contrast, control grapevines and 5YO samples were mainly positioned in the upper part of the ordination, where they were associated with soil pH, soil moisture, petiole sulfur concentrations, and several ‘bulk-soil’ enzymatic activities, including BG, CBH, NAG, and XYL.

Overall, the RDA indicates that the presence of trees was associated with shifts in microbial functional dynamics, particularly in rhizosphere enzymatic activities. The ordination further suggests that these responses in the activity of microbial communities were more pronounced in the vicinity of older trees, highlighting a potential influence of tree age on belowground microbial functioning.

Discussion

Using a field-based vitiforestry system under commercial production conditions, we investigated how the presence and age of trees influences soil physicochemical properties, microbial enzymatic activities, nutrient acquisition strategies and grapevine physiological traits in both bulk soil and rhizosphere compartments. Consistent with our hypotheses, older trees enhanced SOC and N contents and promoted higher enzymatic activities in the grapevine rhizosphere, together with a shift toward greater relative investment in P acquisition. Younger trees generally displayed intermediate responses, suggesting the progressive establishment of tree-driven belowground effects over time. These findings highlight the importance of tree age and rhizosphere processes in shaping belowground functioning in vineyard agroforestry systems.

The presence of trees enhances soil organic matter accumulation and rhizosphere microbial functioning

Consistent with our initial hypothesis (H1), the presence of trees enhanced SOM content and stimulated microbial functioning, particularly in the grapevine rhizosphere. Soils located near older trees exhibited higher SOC, SOM and TN concentrations than soils associated with control grapevines, while the grapevine rhizosphere near trees displayed higher enzymatic activities involved in C, N, and P acquisition. As observed in other agroforestry systems, the increase in SOC and TN observed near older trees is likely a result of the progressive accumulation of litter inputs, pruning residues, root turnover and rhizodeposition (Cardinael et al., 2018; Lorenz and Lal, 2014; Udawatta and Jose, 2011). Trees may additionally contribute to nutrient uplift processes through deep rooting, redistributing nutrients from deeper soil horizons toward surface layers via litter deposition and root turnover (Maeght et al., 2013; Germon et al., 2020). The enrichment in SOM and nutrients observed near trees may create favorable conditions for microbial metabolism and stimulate nutrient cycling (Mooshammer et al., 2014; Fanin et al., 2019; Philippot et al., 2024; Li et al., 2024; Blondel et al., 2025). Over longer temporal scales, trees

may additionally buffer soil temperature and moisture fluctuations, thereby potentially promoting extracellular enzyme activity during periods of environmental stress or reduced water availability (Zuccarini et al., 2023). In the present study, enzymatic activities measured in bulk soil showed relatively limited variations among treatments despite detectable differences in soil physicochemical properties. Such results may reflect the spatial heterogeneity of bulk soil environments, where localized biological responses may remain less apparent than in highly active microsites such as the rhizosphere (Kuzyakov and Blagodatskaya, 2015; Bilyera et al., 2020). In contrast, the grapevine rhizosphere exhibited markedly higher enzymatic activities near trees. The rhizosphere is widely recognized as a hotspot of microbial activity driven by rhizodeposition and root exudation, which stimulate microbial metabolism and enzyme production through the release of sugars, amino acids and signaling compounds (Philippot et al., 2013; Kuzyakov and Razavi, 2019). Grapevines grown near trees may experience altered rhizosphere functioning either because improved nutritional conditions stimulate root exudation (Bowsher et al., 2018) or because tree-induced changes in soil organic matter favor distinct microbial assemblages and functional strategies (Staszal-Szlachta et al., 2024).

Interestingly, the difference in enzymatic activities observed in the grapevine rhizosphere was not associated with detectable differences in DNA content, which is considered as a proxy to evaluate the microbial biomass in soil samples. Such responses are consistent with the idea that rhizosphere-associated microorganisms can rapidly adjust their functional investment to changing substrate availability and nutrient demand without substantial changes to the biomass (Maxwell et al. 2020). It should nevertheless be noted that total extracted DNA may also include extracellular or residual DNA originating from non-living cells, and therefore represents an integrative proxy of microbial biomass rather than a direct estimate of the active microbial community (Nannipieri et al., 2017; Fornasier et al., 2014).

Stoichiometric analyses further support this interpretation. In bulk soil, vector angles increased near older trees, indicating a greater relative microbial investment in P acquisition compared with C and N acquisition (Moorhead et al., 2016; Fanin et al., 2016). This shift occurred despite limited differences in measured soil P availability, suggesting changes in microbial nutrient acquisition strategies that were not directly reflected by bulk soil P pools. Increased root inputs and enhanced microbial processing of organic substrates may therefore have stimulated microbial growth potential and metabolic activity, resulting in a greater relative investment toward P-acquiring enzymes. This interpretation is also consistent with the frequently reported decoupling between bulk soil nutrient pools and microbial nutrient demand, particularly in biologically active microsites such as the rhizosphere (Sanaullah et al., 2016). In contrast, the vector length remained relatively stable among treatments in bulk soil, suggesting no major differences in relative microbial investment toward C acquisition at the whole-soil scale. In the rhizosphere, however, vector length was significantly higher near 5YO trees, indicating a greater relative microbial investment toward C acquisition in this condition. This pattern may reflect localized shifts in rhizosphere microbial activity and C turnover associated with younger trees, whereas the response near 10YO trees appeared less pronounced. Despite this localized increase, the generally moderate vector lengths observed across treatments suggest no major shift toward C acquisition relative to nutrient

acquisition, potentially due to continuous C inputs from root exudation and litter deposition. Overall, these results suggest that the presence of trees influenced not only the magnitude of microbial activity, but also the balance of microbial nutrient acquisition strategies, progressively shifting microbial functioning toward stronger P acquisition requirements as the tree age increased.

Tree age gradually strengthens belowground functional responses

In agreement with our second hypothesis (H2), the effects of trees on soil functioning became stronger as the tree age increased. In most cases, 5YO trees induced intermediate responses between control vines and 10YO trees, suggesting a progressive establishment of tree-driven belowground interactions over time. These temporal patterns were particularly evident for SOC accumulation, rhizosphere enzymatic activities and microbial nutrient acquisition strategies, indicating that the duration of tree-vine co-cultivation plays a major role in shaping belowground functioning in vitiforestry systems. The gradual increase in soil organic matter observed with tree age is consistent with previous studies showing that significant SOC accumulation in agroforestry systems generally occurs only after several years or decades of co-cultivation (Oelbermann et al., 2006; Pardon et al., 2017; Cardinael et al., 2018). Such delayed responses are consistent with the long lifespan and perennial nature of vitiforestry systems, in which biological interactions gradually develop with years of co-cultivation. During the first few years following tree establishment, litter production, root turnover and rhizodeposition remain relatively limited, often resulting in weak or undetectable changes in soil properties (Clivot et al., 2020). Older trees may cause stronger modifications to the soil structure, nutrient availability, microclimatic conditions, and root interactions, thereby gradually altering microbial functional strategies in the vicinity of grapevine roots. These results emphasize the importance of considering the temporal dimension of tree-soil-vine interactions when evaluating the ecological functioning and agronomic benefits of vineyard agroforestry systems.

Improved grapevine nitrogen status without detectable negative impacts on vine productivity

Consistent with our third hypothesis (H3), grapevines planted near trees, particularly 10YO trees, showed an improved nitrogen status without reductions in yields. Although petiole N concentration and must nitrogen content did not differ significantly among treatments, NBI values were positively correlated with both petiole N concentration and ammoniacal nitrogen in must, supporting the hypothesis of enhanced nitrogen acquisition near trees. This pattern may be due to enhanced microbial activity in the rhizosphere, since extracellular enzymes involved in nutrient cycling can increase organic matter mineralization and nutrient availability (Sinsabaugh et al., 2008; Schimel and Schaeffer, 2012). Trees also help to improve soil structure and promote the accumulation of organic matter, indirectly supporting grapevine nutrition.

Some studies conducted in European agroforestry systems have reported increasing competitive effects of trees on crop yields after approximately 10 years in alley-cropping systems (Ivezić et al., 2021). Other

studies have shown that crop diversification has no significant impact on crop yields (Tamburini et al., 2020). These effects appeared to be highly dependent on the type of agroforestry system, climatic conditions, geographical context, and management practices. To our knowledge, the impact of trees on vineyard yields, whether they are located within vine rows or in hedgerows, has never been reported in viticultural systems. In the present study, no clear negative effects on grapevine physiological performance were observed. Interestingly, although several indicators suggested improved nitrogen status near trees, these changes were not associated with measurable differences in grapevine yield components.

Some modifications in berry must composition were nevertheless observed near older trees, including higher tartaric and gluconic acid concentrations and consequently higher total acidity. These results suggest that the presence of trees may also influence grapevine metabolism and berry composition.

In grapevines, must $\delta^{13}\text{C}$ is widely used as an integrative indicator of seasonal water status and water-use efficiency, with higher $\delta^{13}\text{C}$ values generally associated with increased water limitation (Gaudillère et al., 2002; Van Leeuwen et al., 2009). Must $\delta^{13}\text{C}$ values ranged from -25.99 to -27.15‰ and did not differ significantly among treatments. These values are consistent with little to no water limitation during the period studied. The absence of treatment effects suggests that agroforestry integration did not substantially alter grapevine water status and provides no evidence for increased competition for water between trees and grapevines. However, these measurements were obtained during the exceptionally wet 2024 growing season in Bordeaux, which likely reduced water limitation and may have masked potential competition between trees and grapevines which could become more pronounced under drier conditions.

Overall, these results suggest that vitiforestry systems can improve grapevine nutritional functioning without negatively affecting vine productivity under the environmental conditions investigated in this study.

Implications for vineyard sustainability and future directions

The increase in SOM and rhizosphere enzymatic activity near older trees suggests that vitiforestry may enhance soil functioning and ecological sustainability in vineyard systems (Cardinael et al., 2020; Boinot et al., 2022). Although trees were historically integrated into European vineyards through systems such as *hautains* (where vines are trained high) or *joualles* (a traditional system in which vines grow on fruit trees, with other crops cultivated between the rows), such practices have declined with viticultural intensification and mechanization (Eichhorn et al., 2006). Modern vitiforestry systems may therefore provide an opportunity to restore some of these ecological functions while maintaining productivity.

The localized responses observed in this study highlight that the rhizosphere is a key compartment mediating tree-vine interactions, consistent with the role of roots in regulating soil biogeochemical processes through rhizodeposition and plant–microbe interactions (Dijkstra et al., 2021). While the total

microbial biomass changed little, shifts in enzymatic activity suggest that trees mainly altered microbial functional strategies rather than overall microbial abundance. Although microbial community composition and root exudation were not directly measured, the results indicate a functional reorganization of rhizosphere microbial communities driven by changes in nutrient availability and demand.

However, it is difficult to generalize these results, and it is important to keep in mind that a number of environmental parameters may potentially influence the responses observed. Multi-year monitoring would therefore be necessary, as climatic conditions may play a major role. For example, it would be particularly valuable to investigate these responses under water stress conditions to better understand the effects of drought on tree-soil-vine interactions, since competition between trees and vines could become more intense under drier conditions, and consequently alter both microbial functioning and grapevine performance (Lang et al., 2019).

Future studies combining molecular approaches such as metabarcoding, metagenomics, or ddPCR with functional studies (enzymatic assays, bioassays in vitro, etc.) could provide a deeper insight into the mechanisms underlying tree-vine interactions. Finally, long-term and multi-season monitoring, including deeper soil horizons, will be necessary to better understand the temporal dynamics of nutrient cycling, microbial processes, C sequestration, and vertical nutrient redistribution in vitiforestry systems.

Conclusions

This study demonstrates that the presence of trees within vineyard rows can substantially influence belowground functioning in vitiforestry systems, particularly after several years of co-cultivation. Older trees enhanced soil organic matter and total N contents and stimulated enzymatic activities involved in nutrient acquisition in the grapevine rhizosphere. The presence of trees also promoted shifts in microbial nutrient acquisition strategies toward greater relative investment in P acquisition. These effects were spatially localized and primarily expressed in the rhizosphere compartment, highlighting the importance of plant-microbe interactions in mediating the impacts of trees on soil functioning. Despite these modifications, the presence of trees did not negatively affect grapevine productivity. On the contrary, grapevines growing near older trees exhibited indicators of improved N status without evidence of strong water competition under the environmental conditions investigated. Overall, these findings suggest that vitiforestry systems may enhance soil biological functioning and nutrient cycling while maintaining grapevine performance. More broadly, these results emphasize the importance of considering both spatial compartmentalization and the temporal dimension of tree-vine interactions when evaluating vineyard agroforestry systems. They further support the potential of vitiforestry as a sustainable management strategy capable of reinforcing soil health, ecosystem resilience, and the long-term ecological functioning of viticultural systems.

Declarations

Acknowledgements

The authors would like to thank the Vignoble Émile Grelier for providing access to the experimental site and for their support during field sampling. We are also grateful to colleagues from the EGFV unit for their valuable assistance with fieldwork. We also acknowledge Elisa Marguerit for kindly providing the Dualex device, and Agnès Destrac for her help with FOSS analyses. This work was supported by INRAE and the Région Nouvelle Aquitaine in the framework of the TETRAE program. We also thank Laurence Denaix for her contribution to securing funding.

Funding

This work was supported by INRAE and the Région Nouvelle Aquitaine in the framework of the TETRAE program.

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Author contributions

Anne Janoueix: Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Writing – original draft. **Nicolas Fanin:** Methodology, Ressources, Writing – review & editing. **Sylvie Milin:** Ressources. **Virginie Lauvergeat:** Conceptualization, Project administration, Validation, Writing – review & editing, Supervision, Funding acquisition. All authors read and approved the manuscript.

Data availability

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

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Figures

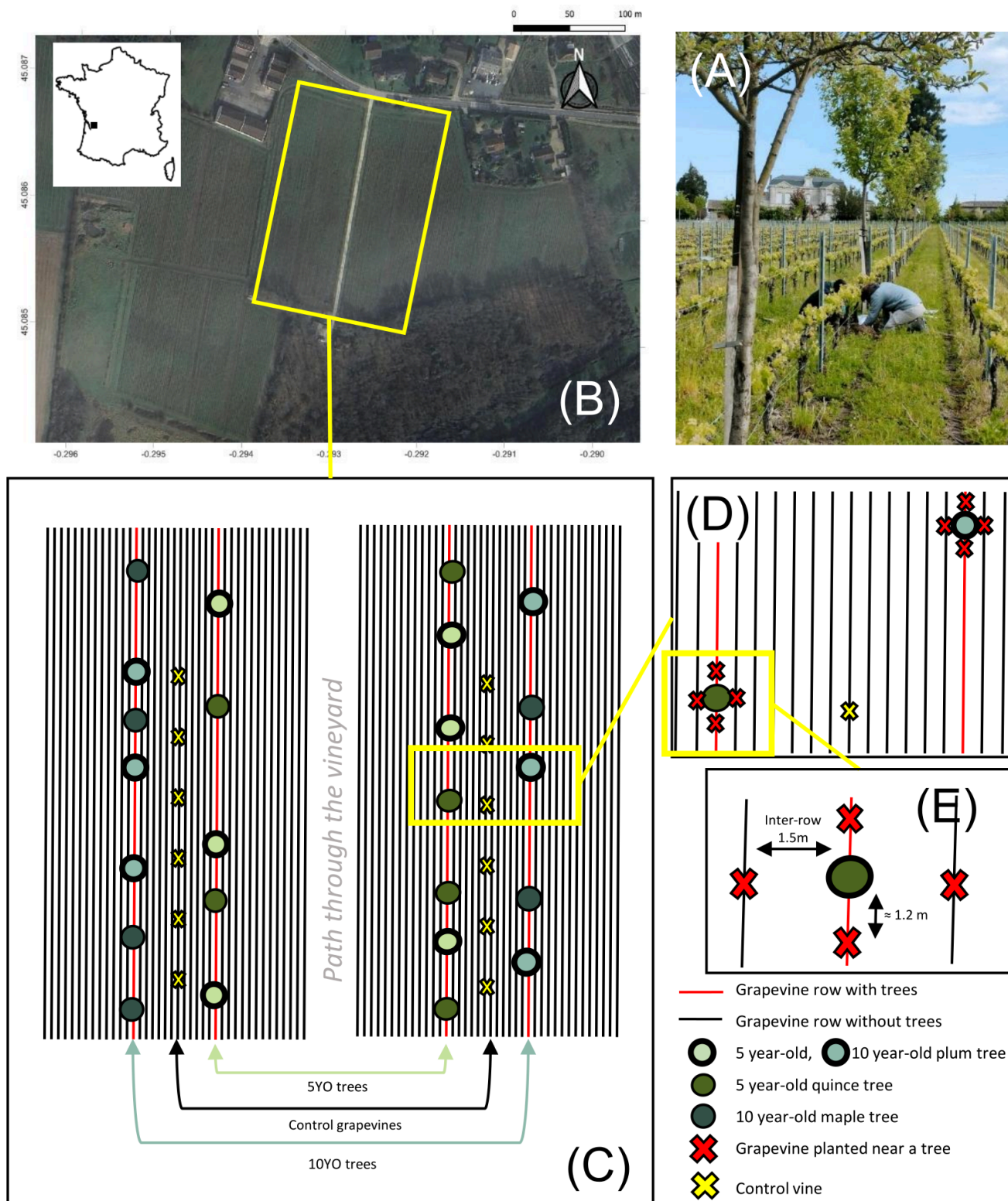


Figure 1

Study site location and experimental design.

(A) Photograph of a grapevine row showing the trees studied during the spring root sampling campaign. (B) Aerial view of the experimental plot. (C) Schematic representation of the experimental design and spatial distribution of the trees studied within the plot. Each black line represents a grapevine row, with

rows spaced 1.5 m apart. Six control grapevines were selected along grapevine rows without trees and located as far as possible from tree rows. (D) Spatial distribution of the sampled grapevines. (E) Illustration of the experimental unit established around each tree.

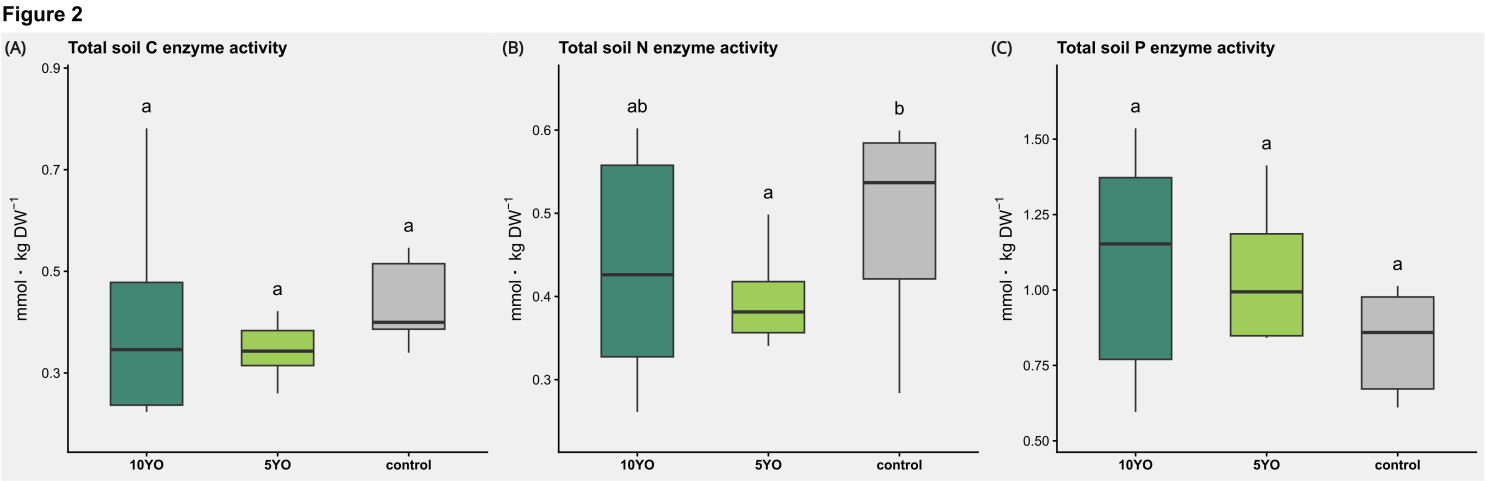


Figure 2

Integrated enzymatic responses measured in bulk soil collected near the plum trees studied (10YO and 5YO) and control grapevines.

Boxplots (n = 6) showing integrated C-related (A), N-related (B), and P-related (C) enzymatic activities expressed as $\text{mmol} \cdot \text{kg DW}^{-1}$. Different letters indicate significant differences among groups ($P < 0.05$).

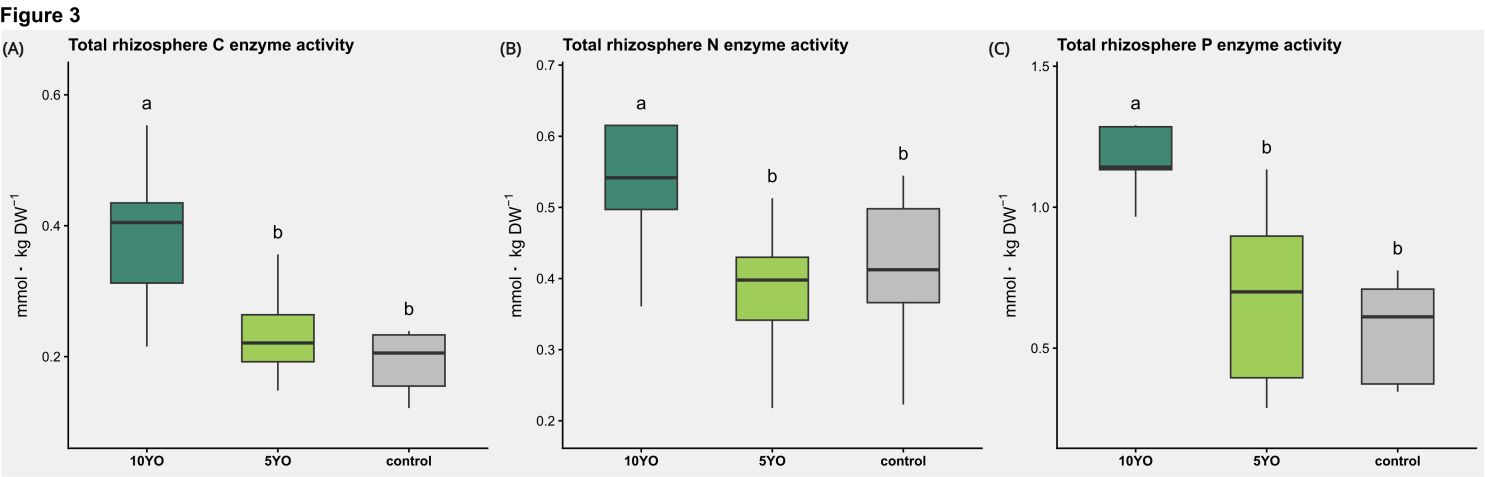
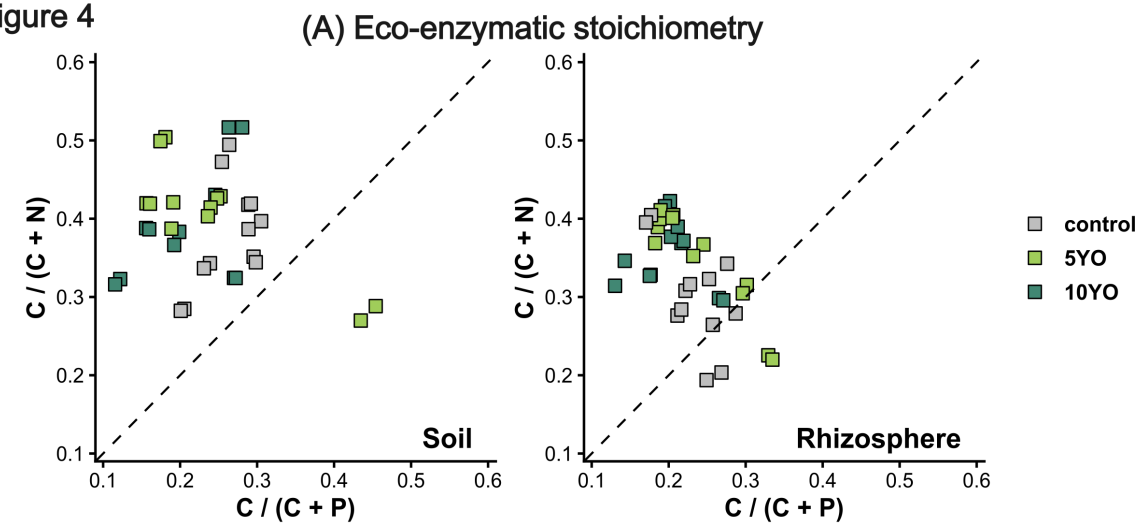


Figure 3

Integrated enzymatic responses measured in the grapevine rhizosphere near the plum trees studied (10YO and 5YO) and control grapevines.

Boxplots (n = 6) showing integrated C-related (A), N-related (B), and P-related (C) enzymatic activities expressed as $\text{mmol} \cdot \text{kg DW}^{-1}$. Different letters indicate significant differences among groups ($P < 0.05$).

Figure 4



(B) Vector length and angle

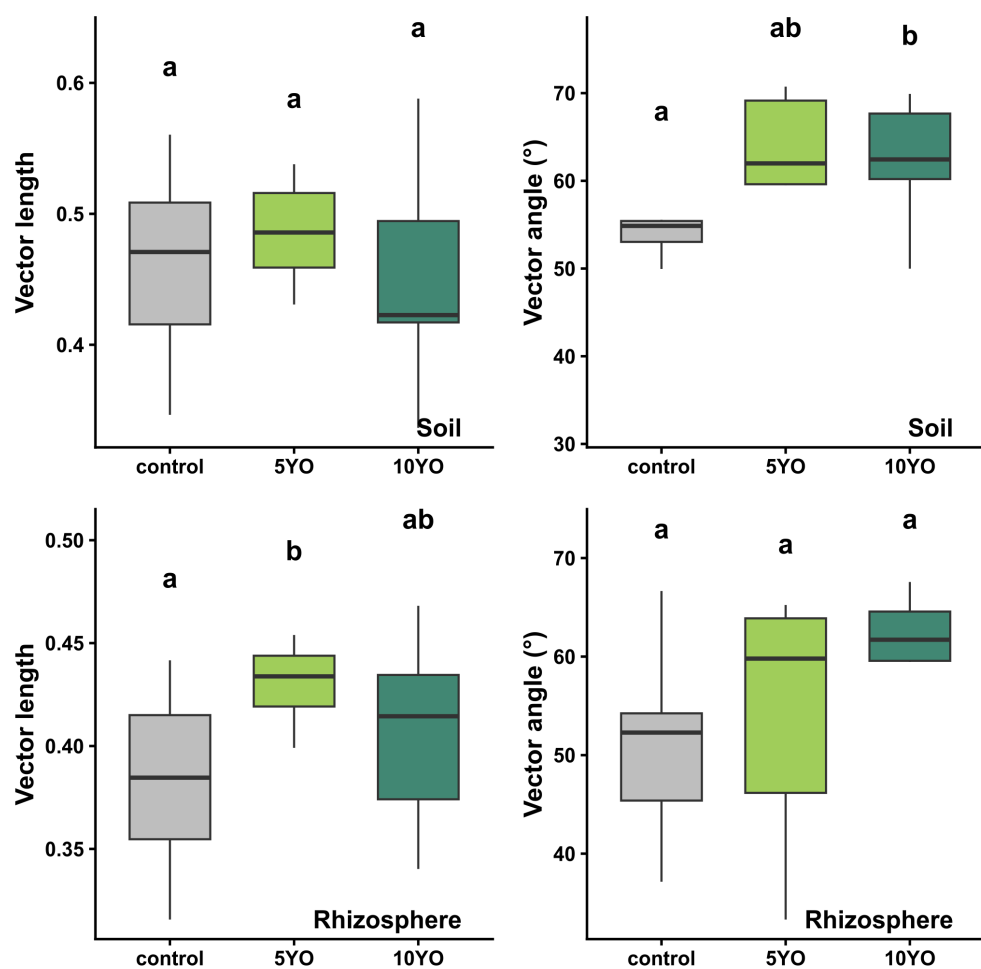


Figure 4

Eco-enzymatic stoichiometry and vector analysis of enzyme activities.

(A) Eco-enzymatic stoichiometry represented as the relative microbial investment in C acquisition compared with P acquisition (x-axis: $C / (C + P)$) and nitrogen acquisition (y-axis: $C / (C + N)$) in bulk soil and rhizosphere samples. Each point corresponds to one sample.

(B) Vector length (left panels) and vector angle (right panels) derived from eco-enzymatic stoichiometry. Upper panels correspond to bulk soil and lower panels to the rhizosphere. Enzymatic pools were defined as follows: C-acquiring enzymes (C) as BG activity, nitrogen-acquiring enzymes (N) as the sum of NAG and LAP activities, and P-acquiring enzymes (P) as AP activity. For each sample, the normalized ratios $C / (C + P)$ and $C / (C + N)$ were calculated and used as coordinates in a two-dimensional space. The vector length was calculated as $\sqrt{x^2 + y^2}$ and reflects the relative microbial investment toward C acquisition compared with nutrient acquisition, with longer vectors indicating stronger relative C limitation. The vector angle was calculated as $\text{atan2}(y, x)$ and reflects the relative allocation of microbial investment between N and P acquisition. Higher vector angles indicate a proportionally greater investment in P acquisition relative to N acquisition, whereas lower angles indicate a shift toward N acquisition. Different letters indicate significant differences among groups based on Kruskal–Wallis tests followed by Dunn’s post hoc tests with Bonferroni correction ($P < 0.05$).

Figure 5

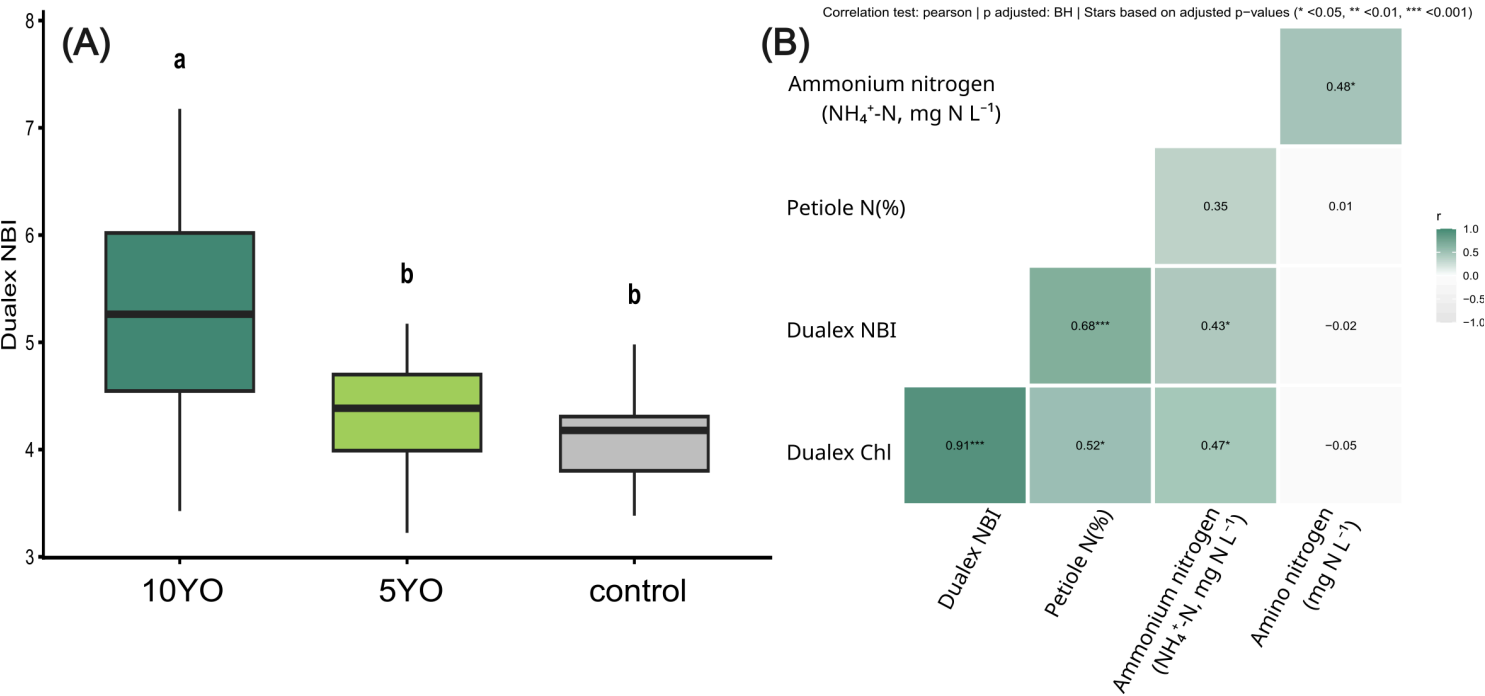


Figure 5

N status of grapevines under different tree-age conditions and relationships among N-related variables.

(A) The Nitrogen Balance Index (NBI) measured with Dualex in grapevine leaves under three conditions: control grapevines, grapevines near 5YO plum trees, and grapevines near 10YO plum trees. Letters indicate significant differences among treatments based on Kruskal–Wallis tests followed by Dunn’s post hoc tests with the Benjamini–Hochberg correction ($P < 0.05$), with $n = 24$ for grapevines near trees and $n = 12$ for control grapevines. (B) The Pearson correlation matrix showing relationships among N-related variables, including the Dualex chlorophyll index (Chl), Dualex NBI, petiole N concentration (%), must ammonium N ($\text{NH}_4^+\text{-N}$, mg N L^{-1}), and must amino N (mg N L^{-1}). Correlation coefficients (r) are

displayed, with significance levels based on Benjamini–Hochberg adjusted p-values (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

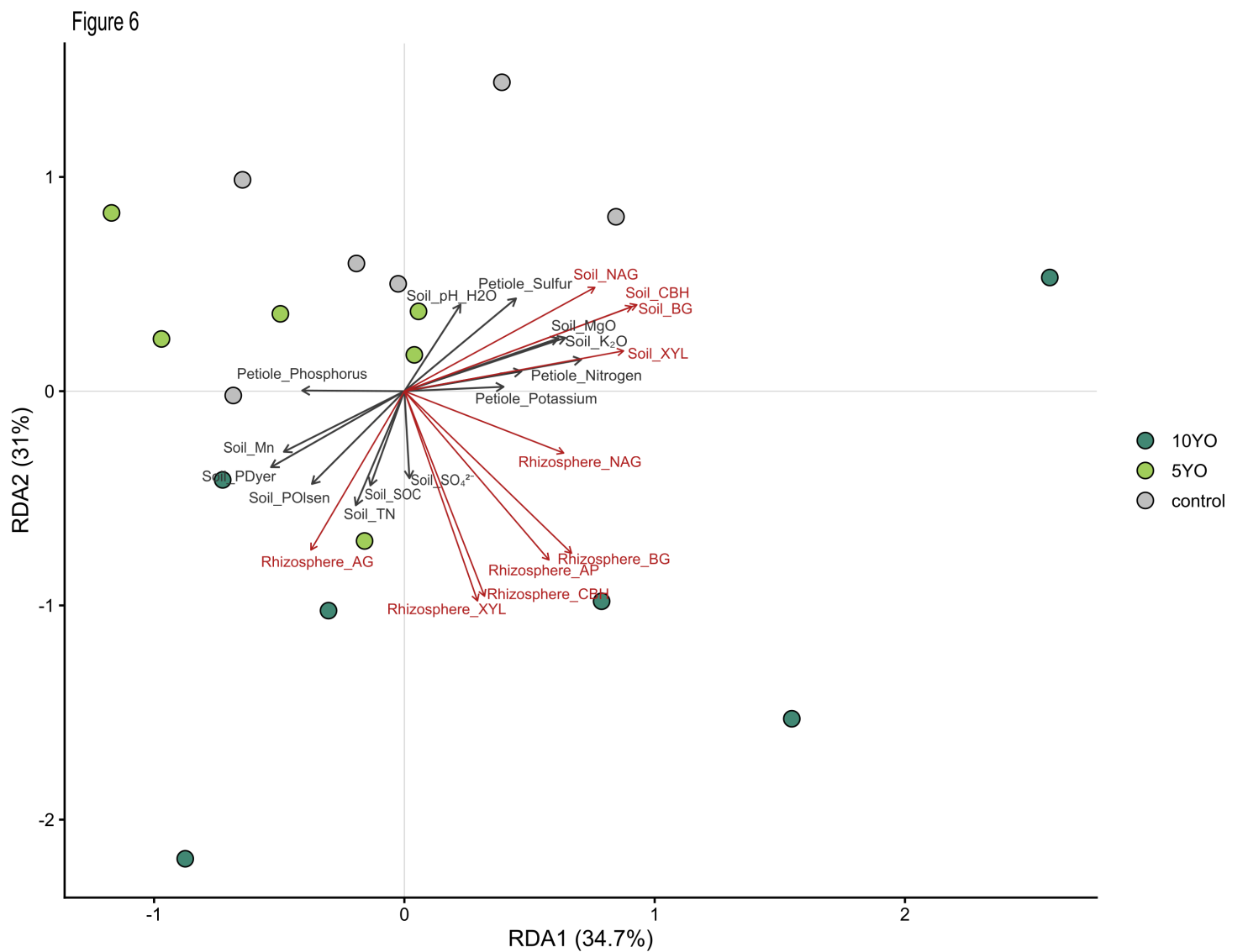


Figure 6

Redundancy analysis (RDA) of enzymatic activities explained by soil physicochemical properties and grapevine physiological variables.

The RDA was performed using standardized enzymatic activity variables measured in bulk soil and the grapevine rhizosphere as response variables, and standardized soil physicochemical properties, petiole mineral status, Dualex measurements, and must composition variables as explanatory variables. Points are colored according to the tree context: grapevines near 10YO plum trees, grapevines near 5YO plum trees, and control grapevines. Grey arrows represent the explanatory variables with the strongest contributions to the first two RDA axes, while red arrows represent the enzymatic variables most strongly projected onto the ordination space. Only the 14 strongest explanatory variables and the 10 strongest

enzymatic variables are displayed for readability purposes. Axis labels indicate the percentage of constrained variance explained by RDA1 and RDA2.

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