



## Analyzing soil enzymes to assess soil quality parameters in long-term copper accumulation through a machine learning approach

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### ABSTRACT

Soil contamination by agrochemicals is a big concern for soil health and ecosystem functioning. This is especially true for non-degradable substances like heavy metals (HM) that, because of their long-term use, are reaching significant values today due to soil accumulation. Among agrochemicals, copper (Cu) has been important in fighting fungal diseases on perennial crops for centuries.

Laboratory experiments can be useful to understand the highest potential toxic effect of Cu but need to reflect what happens with long-term application in a dynamic and living agroecosystem. This study uses multivariate data analysis and machine learning to investigate long-term Cu accumulation on soil quality parameters, especially soil extracellular enzymatic activities. We collected soil samples from 21 apple orchards in South Tyrol, Italy. The orchards had different concentrations of Cu. We took 315 samples in total and analyzed them for various soil properties. We also measured the concentrations of elements in apple leaves and the activities of soil extracellular enzymes. We depicted the effect of Cu on several enzymatic activities, shedding light on the effect of Cu on the soil microbial communities functionality. Our results show that Cu concentrations in the study area affect only phosphatase activity, showing effects above 60 mg kg<sup>-1</sup> of available Cu. Protease activity was positively correlated with Cu, while soil organic matter and management mainly influenced the carbon (C) cycle enzymes. Phosphatase decrease could be of concern for the potential disruption of the Phosphorus (P) cycle in the soil and plays a role in plant nutrition, as seen by P concentration in apple trees' leaves.

We demonstrated how machine learning can help interpret complex and multivariate environmental data and overcome some downsides of traditional statistical models.

### 1. Introduction

Soil degradation resulting from agricultural practices, such as the use of pesticides and fertilizers, is a growing concern due to the presence of heavy metals (HMs) (Guo et al., 2018; Panagos et al., 2018). Many pesticides that were widely used in the past and are still used today contain high levels of metals (e.g., Mancozeb (National Center for Biotechnology Information, 2021)). For example, copper (Cu) containing fungicides such as Bordeaux mixture (Cu sulfate) and Cu oxychloride have been used to protect perennial crops from infections (Cesco et al., 2021). Nevertheless, HMs contamination, mainly Cu, can affect soil

biodiversity particularly by reducing the soil microbial enzyme activities (Karimi et al., 2021; Signorini et al., 2023). As a result, soil enzymes are widely used as indicators of soil health due to their strong correlation with soil quality (Hassan et al., 2022). Also, they are significantly influenced by the different agricultural practices such as irrigation, application of inorganic fertilizers and organic amendments, and soil tillage (Karimi et al., 2021). They present several advantages as indicators, such as operationally practical, sensitive, integrative, measurable, practical, and economical (Utobo and Tewari, 2015). However, certain limitations are associated with soil enzyme activities, including that only potential activity can be assessed, making it challenging to

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distinguish between the background activity of soil-bound enzymes and that originating from soil organisms and plant roots. Therefore, they must always be considered in conjunction with other biological and physicochemical measurements to diagnose soil health correctly (Alkorta et al., 2003).

Previous studies assessing the effect of Cu on soil quality have mainly been conducted in controlled conditions (Karimi et al., 2021). Laboratory experiments on Cu toxicity in soil are short-term and involve an abrupt change in Cu concentration by spiking the soil, thus investigating the so-called acute metal contamination. Soil process responses produced in short-term assays (acute toxicity or disturbance) can be useful to understand the potential toxic effect of Cu but do not reflect what happens in the field with long-term application in a dynamic and living agroecosystem (chronic toxicity or stress) (Giller et al., 2009; Hagemann et al., 2015; Renella et al., 2002). Field experiments are the only way to assess the real effects of HMs on microbial communities, and their results can diverge from laboratory experiments. Such experiments are scarce in literature, and, to our knowledge, very few studies have been conducted in large intensive agricultural districts so far (Schoffer et al., 2022). Our study aimed therefore to explore the impact of soil Cu on enzymatic activities linked to biogeochemical cycles using a machine learning approach to enhance the depth and precision of soil quality investigation. In our investigation, we explored various sites exhibiting a gradient in Cu concentration. We hypothesized a possible influence of Cu on six enzymes (i.e. exoglucanase,  $\beta$ -glucosidase, exochitinase, phosphatase, protease, and arylsulfatase) linked to carbon (C), nitrogen (N), phosphorus (P), and sulfur (S) cycles (Adetunji et al., 2017). We supposed long-term Cu accumulation affects enzymatic activity in agricultural soils, with potential differences in field conditions compared to laboratory settings. We also assessed nutrient content in apple leaves to investigate a possible link between the activity of soil enzymes and plant nutrient content (Dussault et al., 2008). To test our hypothesis, we used machine learning to analyze the gathered data and reveal the possible relationship between soil characteristics and enzymatic activity. Machine learning algorithms, including non-linear and non-parametric ones like Random Forest (RF), are known to be well-suited for handling the complexity of soil physical-chemical data (Hu et al., 2016). Despite their potential, using machine learning methods to assess soil quality is relatively limited within soil science, with only a

few reported examples (Liu et al., 2016; Parent et al., 2021; Paul et al., 2020). Due to the multivariate and nonlinear nature of soil ecological data due to the complex interactions among soil properties, machine learning can enhance our understanding of the key drivers of enzymatic activity in soils. The multivariate and nonlinear characteristics of soil ecological data, arising from complex interactions among soil properties, make machine learning valuable for gaining deeper insights into the key drivers of enzymatic activity in soils. Our overarching goal is to contribute to a better understanding of the impact of chronic Cu contamination on soil health and to demonstrate the applicability of machine learning techniques in soil science research.

## 2. Materials and methods

### 2.1. Study area

This study focuses on apple orchards at the valley floors and sidehills of the Venosta/Vinschgau and Adige/Etsch valley in Bolzano/Bozen, South Tyrol, Italy (Fig. 1). South Tyrol is in the north-east of Italy on the southern side of the main alpine ridge and borders Switzerland and Austria. The area has a typical continental climate with low annual precipitation (450–850 mm) and has a large elevation gradient, from 200 to 3900 m a.s.l. Annual mean air temperature in apple growing sites is between 9.9 °C (Silandro/Schlanders) and 12.3 °C (Bolzano/Bozen). South Tyrol is the largest apple-growing region in Europe, with over 18,000 ha and represents 50 % of the apple production in Italy and 10 % in the European Union (South Tyrol Apple Consortium, 2019; Variety Innovation Consortium South Tyrol, 2021). In this region, apple cultivation has a long tradition and goes back to the 16th century (Variety Innovation Consortium South Tyrol, 2021). Nowadays, apples grow from 200 m a.s.l. up to 1000 m a.s.l. (Variety Innovation Consortium South Tyrol, 2021) and 10 % are managed organically, whereas 90 % are managed with integrated pest management (Fig. 2) (South Tyrol Apple Consortium, 2019). Most organically produced apples are cultivated according to Bioland standards (South Tyrol Apple Consortium, 2019). Most apple varieties in South Tyrol are grafted on dwarfing rootstocks like M9 (Waldner, 2012). M9 T337 is the most common one, followed by scions of M9 like Nic.29 and Pajam 2. In apple orchards, there are from 3,000 up to 6,000 plants per ha (Waldner, 2012), planted in a 2.8–3.2 ×

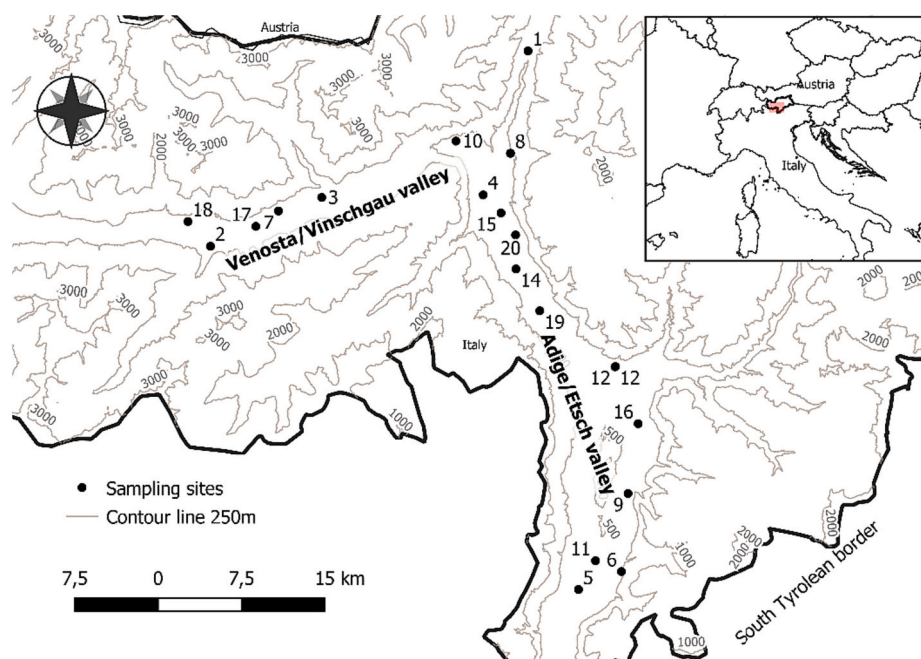


Fig. 1. Study area, main valleys of South Tyrol, Italy. Black points represent sampling sites among which 15 soil samples and 15 leaves samples were collected.



**Fig. 2.** Organic apple orchard (right), Conventional apple orchard (left). Conventional management periodically uses herbicides for weed control, while in organic management mowing is applied. This creates a difference, especially in the tree understory, where in organic management there is a permanent presence of living roots which is lacking in conventional management.

1.2–0.7 m system. Lower plantation densities can be achieved with Bibaum (2.8–3.2 × 1–1.4 m). In South Tyrol, apple trees are trained on a trellised system to a slender spindle. Such plantations are put into the ground for 15 or more years (sometimes even 30 years are possible).

## 2.2. Soil and leaves sampling

The study was conducted across 21 apple orchards. Sampling was performed in June 2020. The 21 sites were chosen to have similar environmental and topographic conditions. In addition, we looked for areas with different land-use history and Cu concentration to have a Cu gradient ranging from 8.18 to 640 mg kg<sup>-1</sup>. The data about soil characteristics were known thanks to previous studies in the area (Della Chiesa et al., 2019a, 2019b; Genova et al., 2021).

Soil and leaf samples were taken from conventionally ( $n = 13$  sites) and organically managed orchards ( $n = 8$  sites). In each apple orchard, soil samples were taken from the topsoil (0–20 cm) of 5 adjacent trees in 3 rows ( $n = 15$  samples) with a hand auger (Eijkelkamp Auger Edelman, Ø 7 cm, a mix of three subsamples compose each sample). Large soil aggregates were crushed, and residues of plant material, roots and stones were removed. A mix of young and old leaves (approximately 10 leaves in total) were taken from each tree under which soil samples were taken. In addition, soil moisture was measured underneath each tree by TDR HydroSense II and averaged.

Soil samples for enzymatic analysis were maintained at 5 °C. Analysis was carried out by a routine soil testing laboratory (Ecorecycling Felderer) according to standardized procedures (see paragraph 2.3 for more details). Soil samples for chemical analysis were the result of mixing all 5 soil subsamples of one row ( $n = 3$  per site).

Leaves were collected based on soil sample locations, then dried at 50 °C and ground using a ball mill (MM400, RETSCH, Pedrengo, BG, Italy). Approximately 0.3 g of each dried sample were mineralized with 4 mL of concentrated ultrapure HNO<sub>3</sub> (650 mL L<sup>-1</sup>) in a single reaction chamber microwave digestion system (UltraWAVE, Milestone, Shelton, CT, USA). After cooling, the digested samples were diluted with Milli-Q water to 20 mL. The following elements were analyzed: Ca, K, Mg, P, Cu, Fe, Mn, Ni, Pb, Zn, and B. The concentrations of elements were analyzed by Inductively Coupled Plasma – Optical Emission Spectroscopy (ICP – OES, Spectro Arcos, Spectro Ametek, Kleve, Germany) using spinach leaves (SRM 1570a) and tomato leaves (SRM 1573a), as external certified reference materials.

## 2.3. Soil physical and chemical analysis

Soil texture was determined by feel test according to the German classification (AD-HOC AG, 2005). Soil pH was determined in 0.01 M CaCl<sub>2</sub> solution and deionized water with a calibrated pH-meter at a soil-to-solution ratio of 1:2.5. Electrical conductivity (EC) was analyzed using conductivity meter (EC-meter, edge™ HI2020–02 HANNA Instruments srl, Italy) with solution ratio of 1:2.5. Total carbon (TC), total nitrogen (TN) and total inorganic carbon (TIC) were assessed using an elemental analyzer (Skalar PrimacsSNC100). For analysis of TC and TN soil samples were combusted at 1400 °C. TIC was determined by mixing the soil sample with phosphoric acid and heating the soil samples at 130 °C. The CO<sub>2</sub> deriving from combustion (TC) or the reaction with acid (TIC) was quantified in a NDIR detector, The N<sub>2</sub> deriving from combustion was quantified by thermal conductivity detection (TCD). The ammonium lactate method according to Egner-Riehm (KAL, pH 3.75) was used to determine plant available K and P. Ca and Mg were also analyzed in the Egner-Riehm extract, which determines the sum of exchangeable Ca, Mg plus the Ca, Mg bound within the reactive carbonate fraction (suffix “Res”, Table S 1). In the experimental soils, the proportion of Ca-res exceeding 500 mg/100 g can clearly be attributed to carbonates; in soils with Ca-res > 800 mg 100 g<sup>-1</sup> soil chemistry is significantly affected by the calcareous conditions (oral communication). The plant available fraction of Mg, Fe, Cu, Mn, Zn and B was analyzed using 0.01 M CaCl<sub>2</sub> and 0.002 M DTPA (diethylenetriaminepentaacetic acid) solution (VDLUFU, 1991) (suffix “CAT”, Table S 1). Water-soluble fraction of Na was determined in deionized water (suffix “Soluble”, Table S 1). Elemental analysis of solutions was conducted by ICP-AES for all soil extracts. Table S 1 shows the soil properties analyzed by the soil testing laboratory (Accredited laboratory under ISO 17025:2005, methods: ISO 10694:1995, DIN EN 15933:2012, ÖNORM L 1087:2012 A.5).

## 2.4. Enzymatic activity essay

Enzymatic activities are either determined by conventional colorimetric methods (Margesin et al., 2002; Tabatabai and Bremner, 1969) or by recently developed fluorescent methods using micro-plate readers, which enable the simultaneous determination of several soil enzymes (Dick et al., 2018). The substrates added are either associated with the highly fluorescent compounds 4-methylumbelliferon (MUF) or 7-Amino-4-methylcoumarin (AMC). The potential hydrolytic enzyme activity of cellobiohydrolase (CBH) (Clayssens et al., 1989; Schwarz et al.,

1987), acid phosphatase (PHO) (Grange, 1978; Neumann, 1948),  $\beta$ -1,4-glucosidase (BGL) (Dick et al., 1990; Sirová et al., 2003),  $\beta$ -N-acetylglucosaminidase (NAG) (Frouz et al., 2003; Sirová et al., 2003), arylsulfatase (AS) (Chiba et al., 1998) and leucine aminopeptidase (LAP) (Sirová et al., 2003) was measured fluorometrically according to (Delledesca et al., 2020) with minor modifications. Soil samples were analyzed 1–3 weeks after soil sampling. Soil suspensions were prepared by placing 1 g soil in a 150 mL beaker adding 100 mL of sodium acetate buffer (100 mmol L<sup>-1</sup> adjusted to pH 5.5 with acetic acid). The soil suspension was homogenized by using an ultrasonicator (Fisherbrand™, Schwerte, Germany) for 1 min at 35 % amplitude. The soil suspension was kept homogeneous using a magnetic stirrer and then transferred to a black microplate well with a pipette. 200  $\mu$ L were transferred to the black microplate well, having 3 replicates for each sample. Fluorometrically labelled substrates Methylumbelliferyl (MUF) and 7-Amino-4-methylcoumarin (AMC) were used to measure enzyme activities.

The enzyme activities measured and the respective substrates and references can be found in the table below (Table 1). Fifty  $\mu$ L of substrate were pipetted into microplate wells with 200  $\mu$ L of soil suspension. To set the calibration, MUF was used for phosphatase, cellulase, sulfatase and chitinase activities, whereas AMC was used for calibration of protease activity. A soil-specific calibration curve was developed for each assay for Leucin-aminopeptidase, AMC was used instead of using 50  $\mu$ L MUF substrate. The samples were then incubated for 3 h at 30 °C in the dark. Relative fluorescence was measured at 365 nm extinction and 450 nm emission with a fluorescence spectrophotometer (Tecan Infinite F200 Fluorometer).

## 2.5. Data analysis and machine learning

For the statistical analysis we relied on ordination techniques to reduce the dimensionality of the multivariate dataset and machine learning to model and automatically find patterns in the data. All the analysis was done with the R programming language (R Core Team, 2021), the data are available in the supplementary material.

Constrained Correspondence Analysis (CCA) was used for dimensionality reduction using the R package *vegan* (Oksanen et al., 2020). CCA is a multivariate method that can reveal the relationships between biological assemblages of species and their environmental variables. A typical use for CCA in the experimental context is to identify the environmental factors that explain the most variation in species composition among different sites. In this study we used CCA to see how the soil chemical and physical analysis vary given the variability of enzymatic activity dataset. The original method was by ter Braak (1986), but the implementation used follows Legendre and Legendre (Legendre and Legendre, 2012). CCA will find linear combinations of two vectors (e.g., X and Y) which have maximum correlation with each other. Harold Hotelling first introduced the method in 1936 (Hotelling, 1936), but in the context of angles between flats the mathematical concept was published by Jordan in 1875 (Jordan, 1875).

**Table 1**  
Measured enzyme activities with their substrates.

Enzyme name	Fluorescent substrate	Enzyme reference	Manufacturer code
Exoglucanase	4-MUF $\beta$ -D-cellobioside	EC 3.2.1.91	CAS No. 72626–61-0
$\beta$ -Glucosidase	4-MUF- $\beta$ -D-glucopyranoside	EC 3.2.1.21	CAS No. 18997–57-4
Exochitinase	4-MUF-N-acetyl- $\beta$ -D-glucosaminid	EC 3.2.1.52	CAS No. 37067–30-4
Protease	Leucine-aminomethylcoumarin	EC 3.4.21	CAS No. 62480–44-8
Arylsulfatase	4-MUF sulfate potassium salt	EC 3.1.6.1	CAS No. 15220–11-8
Phosphatase	4-MUF-phosphate	EC 3.1.3.2	CAS No. 3368-04-5

We employed a dual approach involving Classification and Regression Trees (CARTs) and RF algorithms to analyze enzymatic activity and uncover patterns and thresholds within soil data. CARTs are a method for creating decision trees through iterative binary recursive partitioning. Initially, all training data records are grouped into one partition, and the algorithm then splits them into two branches based on the best binary split for each field, minimizing the squared deviations from the mean. This process continues until nodes reach a specified minimum size or have zero squared deviations from the mean. On the other hand, RF is a machine-learning technique that leverages ensemble learning by combining multiple decision trees. This ‘forest’ is trained using bagging (bootstrap aggregating) and makes predictions by averaging the outputs from various trees. RF models tend to perform well on unseen data and are less susceptible to outliers, but they are computationally intensive and do not offer a straightforward visual representation. To strike a balance, we fitted and interpreted six CARTs and six RF models, each corresponding to a specific enzymatic activity listed in Table 1. Additionally, we used a linear model to investigate the potential impact of Cu on leaf nutrient content Table 1.

## 3. Results and discussion

### 3.1. Exploratory statistics

Table 2 shows the summary statistics of the soil variables of the analyzed samples. Among these, SOM varies from a minimum of 2 % to a maximum of 8.75 %, with a median of 3.95 %. pH ranges from very strongly acidic to slightly alkaline. Cu has a median of 31.76 mg kg<sup>-1</sup>, the third quartile is 92.53 but the maximum value reaches 641.08. Fig. 3 shows the results from the CCA. On the first axis (CCA1) Cu is one of the predominant constraint variables, positively correlated with protease and negatively correlated with phosphatase. SOM, N, Ca, and pH are predominant on CCA2, the first two being positively correlated with exoglucanase and B-glucosidase and negatively correlated with arylsulfatase, while an opposite correlation can be seen for Ca and pH. These are first descriptive clues that Cu may affect protease (positively) and phosphatase (negatively) while SOM and N could be more influential on other enzymatic activities such as the carbon-cycle related ones. pH has low loading values on both CCA1 and CCA2 and it is therefore not useful to describe the enzymatic activity on these axes. The CCA coefficients for the soil variables can also be found in Table S 2.

Fig. 4 refers to the correlation matrix between the soil properties and the enzymatic activity. Here we see how pH and Ca have a strong positive correlation and are both negatively correlated with Fe. SOM and N also show a strong positive correlation with each other. Boron shows a positive correlation with SOM and Mg, Zn is positively correlated with Cu, Mn does not show strong correlations with other elements and so goes for Al. K is positively correlated with Conductivity. Na is positively

**Table 2**  
Summary statistics of the soil variables analyzed in this study.

Parameter	Unit	Median	Mean	SD
SOM	%	3.95	4.19	1.44
N tot	%	0.23	0.25	0.09
pH	–	6.65	6.59	0.64
Conduct	$\mu$ S/cm <sup>-1</sup>	197.5	217.06	90.79
Mg CAT	mg kg <sup>-1</sup>	30.5	34.35	12.02
B CAT	mg kg <sup>-1</sup>	0.07	0.08	0.02
Fe CAT	mg kg <sup>-1</sup>	13.02	12.95	5.93
Cu CAT	mg kg <sup>-1</sup>	3.18	10.83	15.7
Zn CAT	mg kg <sup>-1</sup>	1.82	2.37	1.65
Mn CAT	mg kg <sup>-1</sup>	3.7	4.42	2.87
Na Soluble	mg kg <sup>-1</sup>	0.88	2.9	3.92
Ca Res	mg 100 g <sup>-1</sup>	440	819.94	759.61
K Res	Mg 100 g <sup>-1</sup>	20	21.41	8.29
P Res	mg 100 g <sup>-1</sup>	50.5	48.22	23.96
Al Res	mg kg <sup>-1</sup>	9.5	10.4	4.1

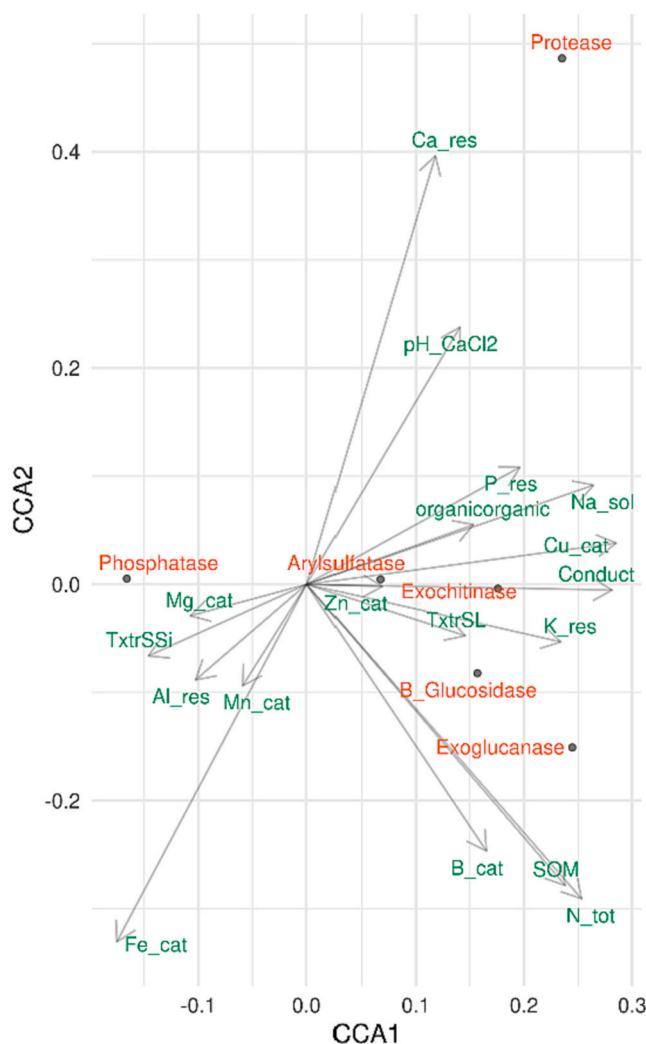


Fig. 3. Constrained Correspondence Analysis for the six enzymatic activities constrained by the soil variables.

correlated with  $\beta$ -glucosidase and exoglucanase. Exochitinase,  $\beta$ -glucosidase, and exoglucanase are positively correlated and with SOM and N. Phosphatase is negatively correlated with Cu and P. Protease is positively correlated with pH and Ca while being negatively correlated with Fe. Arylsulfatase is positively correlated with SOM and N while negatively correlated with P. These correlations and the patterns observed in the CCA will be used as support for the following paragraphs.

### 3.2. Random forest and regression trees

#### 3.2.1. Carbon cycle enzymes

From the RF model (Fig. 5, Table 3), we see how the enzymatic activities dealing with the carbon cycle, (namely exoglucanase,  $\beta$ -glucosidase, and exochitinase) not only share the same most important variables such as SOM and N, but also slightly differ in terms of which variables appear to be the most important and for their position on the ranking of relevance. These enzymes follow similar patterns in the regression trees as well (Fig. 6). When SOM is higher than 4.55 % (exoglucanase and  $\beta$ -glucosidase) or 4.85 % (exochitinase) the activity is higher than the average. Among the high-SOM samples, orchard management (organic or non-organic) plays a role in showing organically managed samples with higher activity levels. Among the low-SOM samples for exoglucanase and  $\beta$ -glucosidase SOM is again identified as the variable influencing the activity (the lower the SOM, the lower the activity), while for exochitinase the electrical conductivity is positively

correlated with the enzymatic activity. Among these regression trees there is no sign of Cu influencing the potential activity of these enzymes. This is confirmed by the RF model, which identifies Cu as a low-importance variable for all the carbon-cycle-related enzymes (Fig. 5). For  $\beta$ -glucosidase this confirms what was seen by (Dussault et al., 2008). Nevertheless, other studies indeed found a negative effect at 450 mg kg<sup>-1</sup> of Cu (Cao et al., 2020). This discrepancy could be due to the relatively lower concentrations of Cu found in our study area (.

Table 2). Moreover, laboratory and field experiments most likely lead to different Cu dynamics and effects on enzymatic activity. In fact, lab experiments mimic acute Cu toxicity while field trials (e.g., sampling soil from real conditions where long-term Cu-containing fungicides have led to Cu accumulation) mimic chronic Cu toxicity. In this study, SOM is what mostly influenced enzymatic activity, and this is confirmed by the literature (Kotrocó et al., 2014). The overall positive effect of soil organic matter on enzyme activities found in our study is in accordance with previous studies (Aponte et al., 2021) and, according to our results, organic farming indeed enhances the potential enzymatic activity but only when SOM is quite high (higher than 4–5 %). The higher enzymatic activity in organically managed soils (when SOM is high) could be caused by the kind of fertilization used, where for instance the application of manure in organic farming can significantly increase hydrolytic enzymes. In addition, previous studies show how the labile fraction of the SOM is rapidly decomposed from the hydrolytic enzymes (Kotrocó et al., 2014).

Litter quantity and quality can have an influence on the enzymatic activity and soil pH, which also has a direct effect on microbial communities and, therefore, on the enzymatic activity (Błońska et al., 2017, 2021; Wang et al., 2020). We see how, in our sites (Fig. 2), the kind and quantity of litter changes dramatically between organic and conventional apple orchards. In Conventional management, herbicides are often used under the trees, and this is where the samples of this study were collected. Conversely, weeds are only mulched and left on the ground in organic agriculture. This leaves more litter, root exudates and dead roots, which are an important source of nutrients for microorganisms, and increase their number and activity (Błońska et al., 2017). This could explain the increased activity in organic apple orchards.

#### 3.2.2. Protease

Protease activity is influenced by pH, Ca, Fe, Cu and B according to the RF model (first 5 most important variables) (Fig. 5), and the CART identifies Ca, Cu and Na as useful variables to describe the variability of the enzymatic activity levels (Fig. 7). Samples having Ca higher than 1128 mg 100 g<sup>-1</sup> have a higher activity, and among these, when Cu is higher than 80.77 mg kg<sup>-1</sup> the activity is around 100 nmol g<sup>-1</sup> h<sup>-1</sup> higher compared to samples with Cu lower than 80.77 mg kg<sup>-1</sup> the samples with low Ca (below 1128) having Na lower than 1.4 is lowering the median values below the overall median. The effect of Cu increasing protease activity confirms what is seen in the CCA, although the mechanism underlying the process is still undefined and unclear. Previous studies drew contrasting conclusions on this matter, as some found inhibition of protease activity in Cu-spiked soils (Effron et al., 2004), while others found no impact on protease activity in soils spiked with Cu-containing nanosized agrochemicals (Tang et al., 2021). In other studies, arsenic did not significantly affect the activity of protease, whereas cadmium significantly reduced protease activity (Lorenz et al., 2006), underlining the effect that the type of heavy metal has on protease activity. Other studies showed no effect of Cu on protease activity (Dussault et al., 2008). The discrepancy between our findings and the previously cited work most likely derives from laboratory and spiking experiments not reflecting in vivo conditions as described earlier. In addition, Protease can vary its activity according to the seasonal period of the year or climatic conditions (Łukowski and Dec, 2018), making it hard to compare different studies. We also see an effect of Ca on protease activity. In this case, we hypothesize an effect of Ca on pH (deriving from carbonates present in certain sites of the study area), which is a key

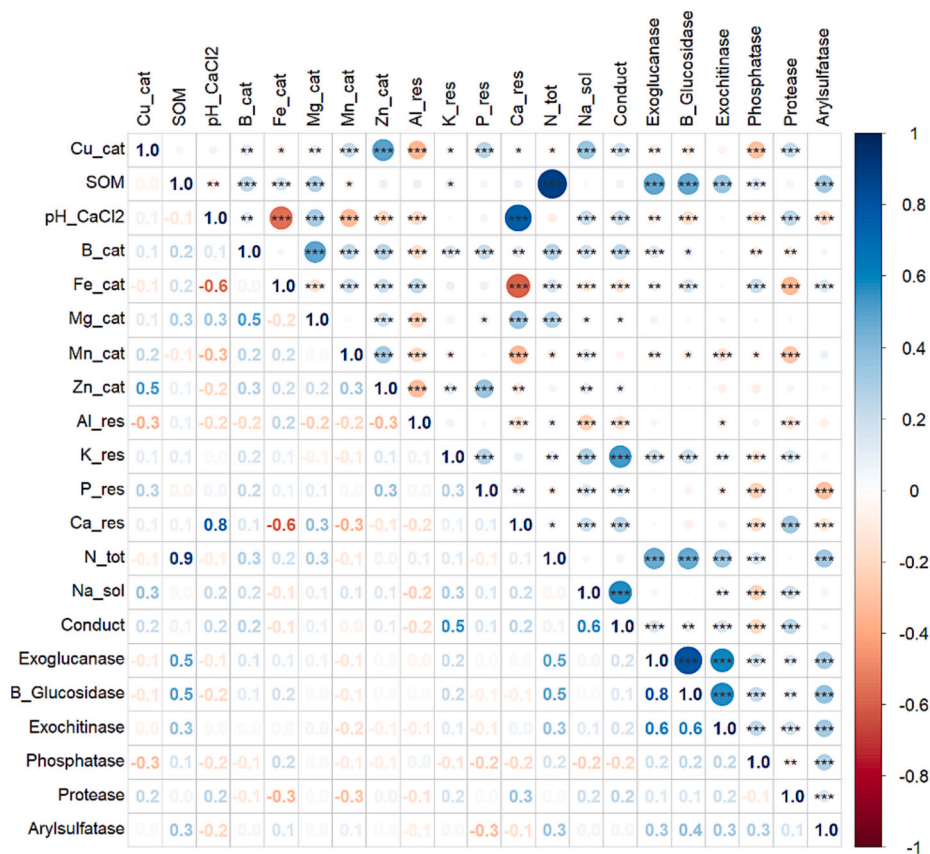


Fig. 4. Correlation matrix considering the soil properties and the enzymatic activity. The Kendall correlation coefficient was used. Both correlation coefficients (bottom part of the matrix) and significance levels (upper part of the matrix) are shown. Signif. codes: 0 ≤ '\*\*\*\*' < 0.001 < '\*\*\*' < 0.01 < '\*\*' < 0.05 < '.' < 0.1 < '<' < 1.

factor for proteases (Neina, 2019).

### 3.2.3. Arylsulfatase

Arylsulfatase is influenced by texture, P, N, SOM, and management (organic or conventional) according to the RF model (first 5 most important variables) (Fig. 5), and the CART identifies N, P and texture as useful variables to describe the enzyme activity variability (Fig. 7). The N content increases the activity when higher than 0.22 %, and P negatively impacts the higher N samples, bringing samples above 52.5 of P mg 100 g<sup>-1</sup> below the overall median. The results for this enzyme could be clearer compared to the other enzymes. The drop in variable importance in the RF model is less sharp for this enzyme (Fig. 5) than the other enzymes, highlighting lower confidence in identifying driving factors for the variability of arylsulfatase activity. Nevertheless, P and N are crucial for arylsulfatase activity (Wang et al., 2019). The texture is important, and sandy soils have relatively low activity. However, the activity levels measured in silty soils, which are just a few in this study, exhibited a high standard deviation, making it difficult to compare their values with those measured in sandy soils, and interpret the results.

### 3.2.4. Phosphatase

According to our RF model, which pinpoints the five most influential variables (Cu, Fe, P, pH, and Na, as illustrated in Fig. 5), and the CARTs analysis, which singles out P, Cu, and Na as pivotal variables in explaining the variability of enzyme activity (as shown in Fig. 7), it becomes evident that these factors play a substantial role in shaping phosphatase activity.

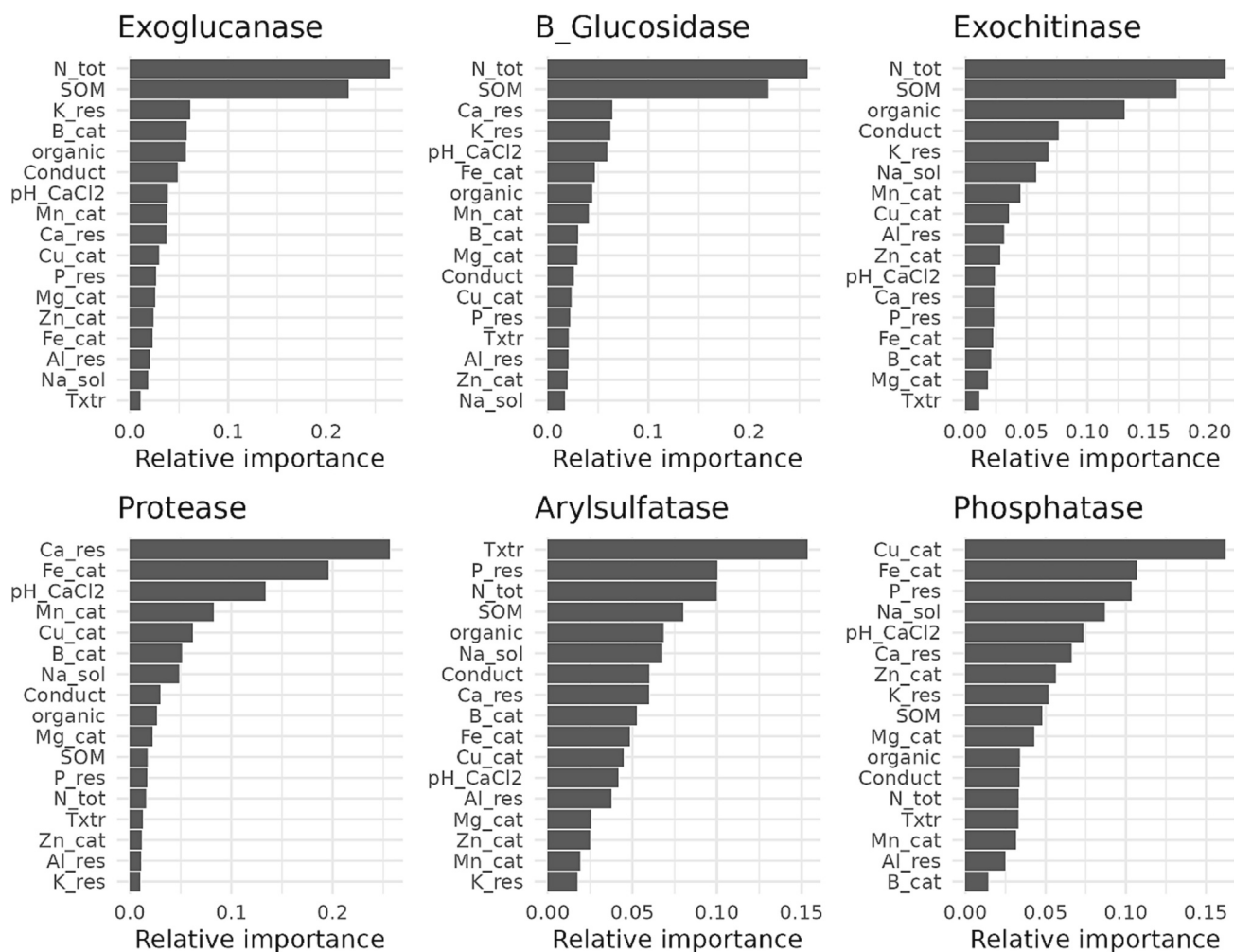
When phosphorus (P) levels exceed 24.5 mg/100 g<sup>-1</sup>, we observe a subsequent reduction in phosphatase activity. Among the subset of samples exhibiting higher P concentrations, the presence of Cu has a negative impact, with samples above 56.6 mg kg<sup>-1</sup> of Cu having

phosphatase activity below the overall median. We can consider this value as a threshold for Cu influence on Phosphatase activity. This observation closely aligns with our grouping of samples based on increasing Cu concentrations, where we indeed notice a significant decrease in phosphatase activity, as corroborated by Fig. S1 and Table S3. We therefore emphasize that our hypothesis regarding the reduction of Phosphatase enzymatic activity in the presence of Cu has been demonstrated.

For samples with low phosphorus levels, sodium (Na) positively influences enzymatic activity when present at levels exceeding 8.3 mg kg<sup>-1</sup>. The inverse relationship between phosphatase activity and phosphorus is likely linked to the “quorum sensing mechanism” (Krupke et al., 2016). When phosphorus is readily available, microorganisms don't need to produce the enzyme to break down proteins to access it. Furthermore, we find that, in cases where phosphorus levels are low, an increase in sodium (Na) content above 8.25 mg kg<sup>-1</sup> enhances enzymatic activity.

These findings align with previous research in winegrowing soils, where phosphatase activity was found to be most sensitive to both total Cu (Cu<sub>total</sub>) and available Cu (Cu<sub>DTPA</sub>) content (Fernández-Calviño et al., 2010). Similar impacts of CaCl<sub>2</sub>-Cu, total Cu, and organic carbon (C<sub>org</sub>) on phosphatase activity in apple orchard soils have been reported (Wang et al., 2009). Additionally, phosphatase has been reported the sole enzyme significantly influenced by Cu in a terrestrial model ecosystem (Lebrun et al., 2012).

To provide a biochemical rationale for the effects of Cu on phosphatase activity, we can rely on the Canonical Correlation Analysis (CCA) (Fig. 3), which is constrained to the enzymatic activity dataset. This analysis shows a positive correlation between phosphatase activity and magnesium (Mg), while Cu displays an inverse correlation with phosphatase activity. It is important to note that Mg is a crucial cofactor



**Fig. 5.** Variable importance for the random forest model for each enzymatic activity considered in this study, the higher the value, the higher the importance for the soil variable. The importance of a variable is computed following the permutation principle of the mean decrease in accuracy importance and then scaled to relative importance.

**Table 3**

Accuracy of the six RF models dealing with enzymatic activity and soil characteristics. They are computed with a test set comprising 15 % of the data.

Enzyme	R <sup>2</sup> squared	RMSE
Exoglucanase	0.8	38
B'Glucosidase	0.8	510
Exochitinase	0.3	74
Protease	0.7	19
Arylsulfatase	0.8	16
Phosphatase	0.5	224

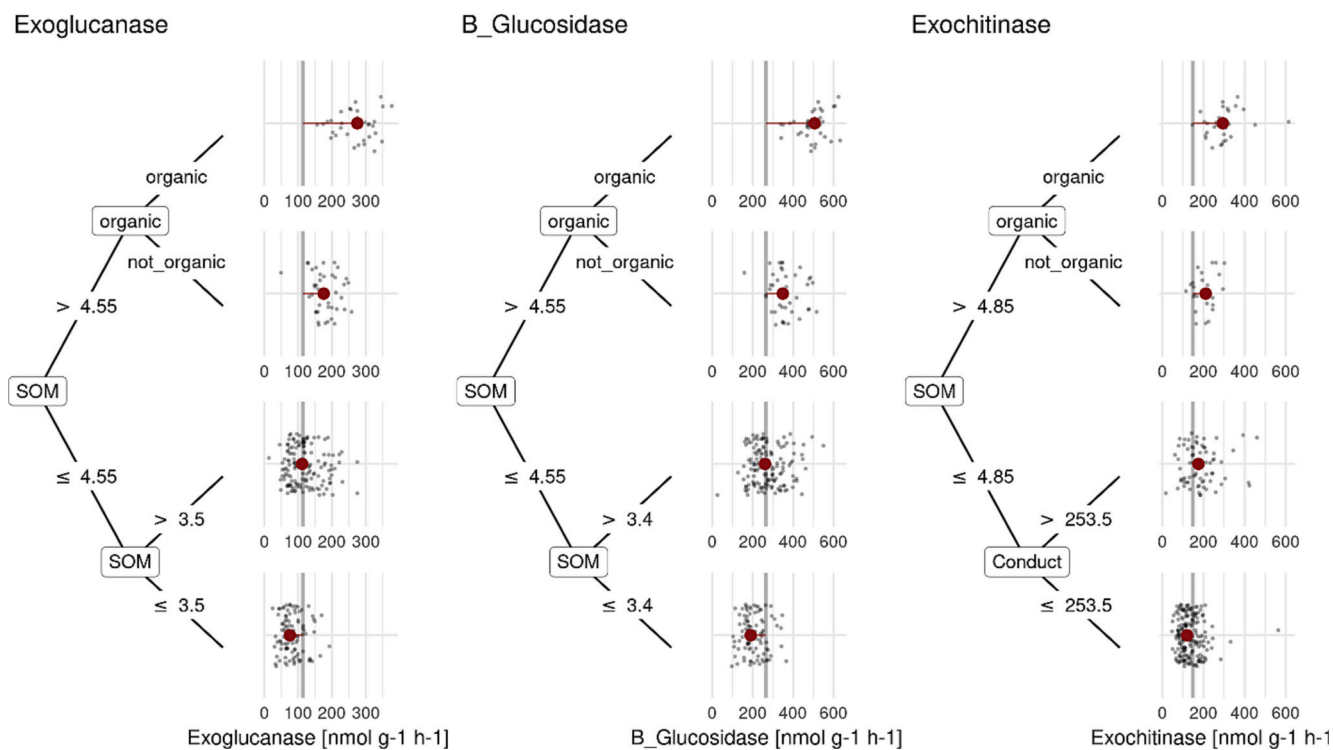
for phosphatase functioning (Anderson et al., 1975; Dean, 2002). Consequently, a plausible biochemical explanation for Cu's impact on phosphatase activity could be the displacement of Mg from the active site due to the presence of Cu, as suggested by (Csopak and Falk, 1970).

Considering these findings, it is evident that Cu exerts a multifaceted influence on phosphatase activity, with its effects intricately intertwined with other vital factors, including phosphorus, sodium, and magnesium. This complex interplay underscores the intricate nature of enzymatic processes within soil ecosystems and emphasizes the imperative need for a comprehensive understanding of these interactions to advance our knowledge in soil science. The threshold of 56.6 mg kg<sup>-1</sup> of Cu on phosphatase activity is automatically derived from the machine learning model and successfully addresses the goals of this study.

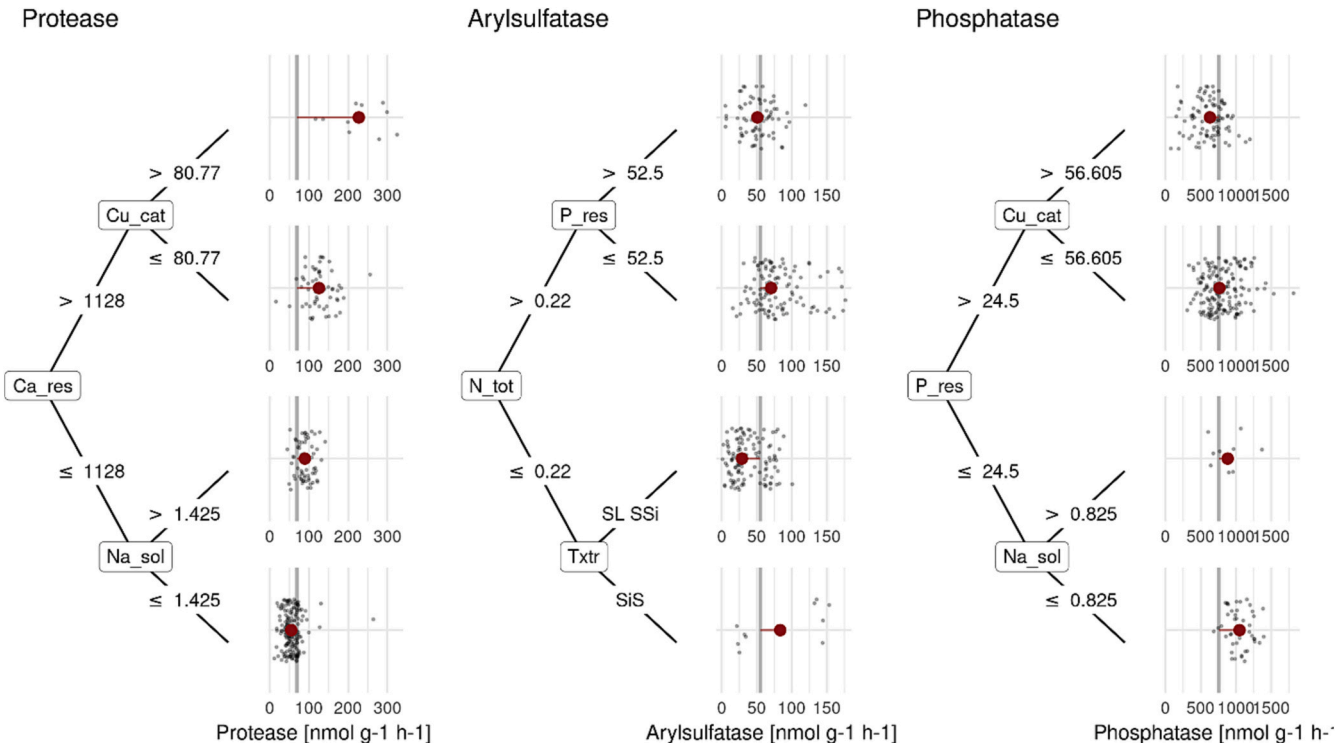
### 3.3. Nutrient content in leaves and Cu in soil

Since phosphatase resulted to be the only enzyme negatively affected by Cu in soil we hypothesized and tested an effect on P content in the apple tree leaves. We also checked other nutrients in the leaves, and we did not find any correlation with Cu (data not shown). The results of a linear model between P in the leaves and Cu in soil can be seen in Fig. 8 and Table 4.

The model depicts a negative correlation between Cu concentration in soil (log transformed) and P in the leaves. The intercept and the coefficient are statistically significant, and the overall model describes a significant trend. The R-squared is low (Adjusted R-squared: 0.1241) highlighting the high variability of the data around the trendline; this could be due to the measurement uncertainty and or to the presence of outliers, but also due to Inherent biological variability of apple leaves across a such broad agronomic district. Nevertheless, we can confirm a possible causal effect between the amount of Cu in soil and the P in the leaves. P in soil is mostly in the organic form and needs to be mineralized (transformed in phosphate) by the soil microbiota to be available for plants (Alori et al., 2017). Cu is negatively correlated with the phosphatase activity (see Sections 3.1 and 3.2) meaning a detrimental effect on the P mineralization. In addition, reduction of shoot phosphorus content could be caused by Cu/P antagonism at the root level (Marastoni et al., 2019).

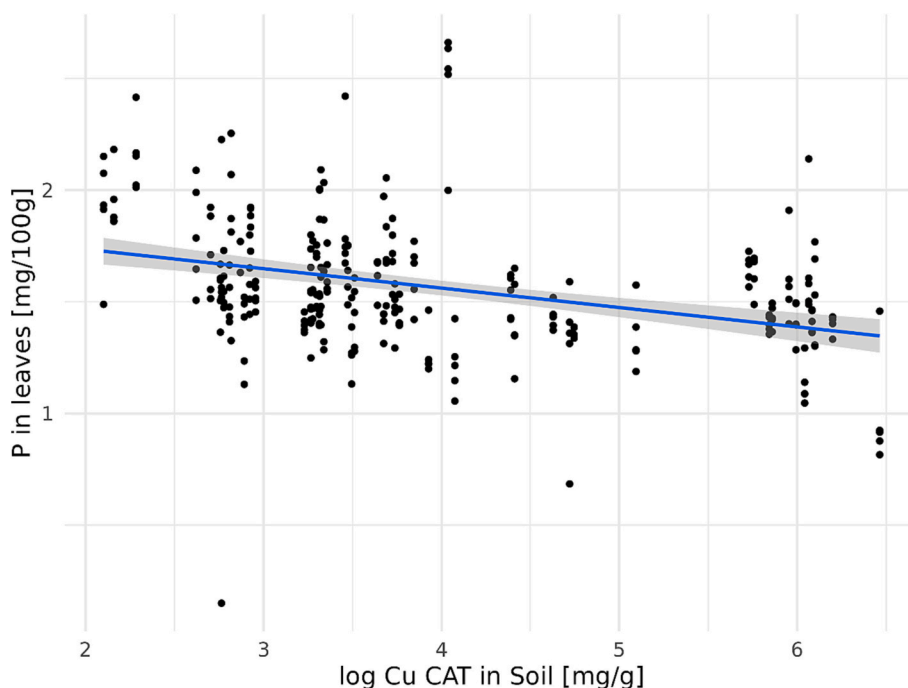


**Fig. 6.** Regression trees for exoglucanase,  $\beta$ -glucosidase and exochitinase activities. Every node represents a split in the dataset that minimizes the sum of the squared deviations from the mean in the two separate partitions. The maximum depth of the regression trees is 2. In the plots of the final nodes the points represent the enzymatic activity value for each sample, the red dot represents the median of the samples and the vertical gray line the median of the entire dataset. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 7.** Regression trees for protease, arylsulfatase and phosphatase activities. Every node represents a split in the dataset that minimizes the sum of the squared deviations from the mean in the two separate partitions. The maximum depth of the regression trees is 2. In the plots of the final nodes the points represent the enzymatic activity value for each sample, the red dot represents the median of the samples and the vertical gray line the median of the entire dataset. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)





**Fig. 8.** Linear model describing the correlation between P in apple tree leaves and Cu in soil (log transformed). The range of Cu concentration of the graph is between 8 and 490 mg kg<sup>-1</sup>.

**Table 4**

Linear model considering P content in the apple trees leaves and Cu in soil.

	Estimate	Standard Error	t value	Pr(> t )	
(Intercept)	1.709	0.028	60.755	0.0000	***
log (Cu'cat)	-0.087	0.014	-6.378	0.0000	***

Signif. codes: 0 ≤ '\*\*\*' < 0.001 < '\*\*' < 0.01 < '\*' < 0.05 < '.' < 0.1 < '<' < 1. Residual standard error: 0.2797 on 279 degrees of freedom. Multiple R-squared: 0.1272, Adjusted R-squared: 0.1241. F-statistic: 40.67 on 279 and 1 DF, *p*-value: 0.0000.

#### 4. Conclusions

In this study, we employed regression trees and random forest techniques to comprehensively analyze the effect of Cu on soil extracellular enzymatic activities in intensively managed apple orchards. Our findings shed light on the intricate relationships among soil composition, including HM concentration, and enzymatic activity particularly focusing on soil enzymes related to C, N, S, and P cycles. Copper primarily disrupted phosphatase activity, potentially through the displacement of Mg ions on the active site. This disruption of phosphatase activity raises concerns about its implications for the P cycle in soil and its effects on plant nutrition, as evidenced by variations in P content in apple tree leaves. Furthermore, the application of machine learning techniques proved to be valuable in navigating the complex interplay between soil properties, pollution, and enzymatic activity. Using this approach, we not only identified Cu as a significant factor in the enzymatic activity but also determined a threshold value for Cu concentration concerning phosphatase activity. These results represent a critical step forward in assessing the potential toxic effects of Cu accumulation in intensive agricultural soils, aligning with the goals of increasingly sustainable agriculture practices. Surprisingly, we uncovered a positive correlation between Cu concentration and protease activity in soil. This unexpected finding challenges existing literature and highlights the need for further elucidate the underlying thresholds and field processes driving this relationship.

In conclusion, our study highlights the effectiveness of data-driven

approaches in uncovering complex soil ecological relationships. It emphasizes the importance of managing Cu in intensive agricultural settings, underscores the role of phosphatase in the P cycle and plant nutrition, and calls for further investigation into the unexpected correlation between Cu and protease activity. The integration of conventional soil fertility indicators with advanced statistical approaches, including machine learning, can aid ongoing efforts to develop crucial information for defining environmentally sustainable agricultural plans. This benefits not only farmers but also decision-makers and stakeholders dedicated to responsible land management.

#### CRediT authorship contribution statement

**G. Genova:** Conceptualization, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **L. Borruso:** Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – review & editing. **M. Signorini:** Investigation. **M. Mitterer:** Investigation. **G. Niedrist:** Conceptualization, Supervision, Writing – review & editing. **S. Cesco:** Investigation, Supervision, Writing – review & editing. **B. Felderer:** Formal analysis, Investigation, Methodology, Writing – review & editing. **L. Cavani:** Methodology, Writing – review & editing. **T. Mimmo:** Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

#### Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Giulio Genova reports financial support was provided by Free University of Bozen-Bolzano. Giulio Genova reports equipment, drugs, or supplies was provided by Eurac Research, Institute for Alpine Environment.

#### Data availability

Data will be made available on request.

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In particular, our study represents an original paper related to the Spoke 4 Multifunctional and resilient agriculture and forestry systems for the mitigation of climate change risks and in particular to the following Tasks:

4.1.2 titled Smart phenotyping platforms for the on-farm selection of resilient varieties and rootstocks (SC);

4.2.1 titled Farm network setup (Living Labs): a network of farms representative of the different agricultural systems to apply innovative technologies for the sustainable management of crops, animals and forests (TM);

4.2.2 titled Advanced monitoring techniques and novel management practices for saving soil and water, optimizing carbon balance, and maximizing the efficiency of used resources and mitigating impacts (TM e LB)

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.apsoil.2023.105261>.

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