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Organic matter evolution and microbial activity in a vineyard soil after four years of inter-row cover crop management

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ABSTRACT

Conservation agriculture approaches are rarely applied to viticulture in central Italy due to some research gaps persisting in this sector that somehow reflect ineffective extension programs and activities. This work analysed the effects on soil health and fertility of two inter-row vineyard management strategies carried out for 4 consecutive years: an annual legume cover crop of Egyptian clover (*Trifolium alexandrinum* L.) and a permanent meadow with spontaneous species. The inter-rows of the two tested strategies were compared with an adjacent uncultivated soil that had been left untilled for 20 years (control soil). The short-term (4 years) impacts of these techniques on the different soil organic carbon forms and on the soil microbial activity were measured at two soil depths (0–20 cm and 20–40 cm). Soil analyses included total and extractable organic carbon, humic and fulvic acids, microbial biomass carbon, respiration and some enzyme tests. The final aim was to evaluate if four years of soil cover could enhance the stabilisation of the soil organic carbon stock and increase the growth and activity of soil microbial biomass.

The permanent meadow showed a topsoil organic carbon content significantly higher than the annual legume cover, and both management soils showed an organic carbon content higher than the control soil. The topsoil humic acid content of the annual legume cover was significantly higher than both the permanent meadow and control, indicating that legume management stored the soil organic carbon in a more stable form. The microbial biomass carbon and the soil respiration of the annual legume cover topsoil were significantly higher than those of the permanent meadow. Regarding the enzyme activities, β-glucosidase and the enzymes related to the nitrogen cycle were significantly higher in the two managements than in the control, while phosphatase activity was not influenced by the two managements.

The results indicate that soil cover in the inter-row of vineyard increases the stable form of soil organic carbon, with variations according to the type of cover adopted. Even the effects on soil microbial biomass growth and activity were positive, especially for the activities related to the carbon and nitrogen cycles.

1. Introduction

A healthy soil, both chemically and biologically fertile, is essential to ensure environmental safety and plant productivity (Brevik and Sauer, 2015; Joyalata Laishram et al., 2013). It is well-recognised that heavy ploughing and other soil disturbances, such as the use of synthetic agrochemicals, could reduce the quality of agricultural soils, negatively affecting both the content and composition of organic matter and the growth and activity of soil microbial biomass (Laudicina et al., 2015; Longa et al., 2017; Mihelič et al., 2024; Yeboah et al., 2016).

In recent years, the diffusion of organic farming as a new agricultural concept that respects the environment and soil health has been promoted in EU countries (European Union, 2018). More recently, the European Union has set an ambitious goal to make all EU soil ecosystems healthy and more resilient by 2050 (European Commission, 2023, 2021). Organic agriculture consists of crop production without the use of synthetic pesticides and fertilisers and with the application of organic amendments to promote increases in both the organic carbon content

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and the biological activity in soils (Angeletti et al., 2021; Domínguez et al., 2014; Li et al., 2019; Monaci et al., 2017). Conservation agriculture is an organic farming system that promotes the use of conservation tillage (i.e. zero- or minimum-tillage) and permanent soil cover to improve soil quality and to allow a sustainable crop production without compromising soil health or resilience (Knapp and van der Heijden, 2018).

According to this approach, the practice of cover crops has been proposed as a conservative intervention to preserve the soil organic carbon (SOC) from degradation and to increase the soil's chemical and biological fertility (Dong et al., 2021; Francaviglia et al., 2017; Gao et al., 2018). These practices are beneficial for perennial crops, such as vineyards, where repeated tillage is commonly used to eliminate competition for nutrients and water, and the use of synthetic pesticides and fertilisers have drastically modified the soil properties and reduced the SOC content. The use of legume cover crops and/or perennial grasses brings more fresh organic matter into the soil and could promote the renewal of soil organic matter and efficiently increase the soil nutrients, enhancing the growth and activity of soil microbial biomass (Agnelli et al., 2014; Gao et al., 2018; Šimanský et al., 2023). In addition, these management practices can protect vineyards from both soil erosion and water loss by runoff, although consociating cover crops with grapevine may also generate both nutrients and water competition depending on local soil and climate conditions (Capello et al., 2020).

Although the potential benefits of conservation agriculture have been proposed and promoted in various sectors, research in the viticulture sector is still relatively limited. This is then reflected in the very low diffusion of these techniques at the level of wine producers.

In order to accurately assess these benefits, it is necessary to monitor and evaluate the evolution of organic matter, both from a chemical and biochemical point of view. After the adoption of these soil management practices, it is crucial to monitor the evolution of organic carbon, particularly its resilience or its disappearance due to the production of labile fractions over time. For example, Ball et al. (2020) investigated the variation in both coarse (> 50 μm) and fine (< 50 μm) SOC fractions from four vineyards in the Adelaide region (Australia), each managed with three different green manure systems, namely grass, legume, and grass/legume mixture. Their results showed that grass consistently increased OC in the total, coarse, and fine pools, probably due to greater root biomass. This should be considered a positive effect since root C significantly contributes to long-term SOC storage. The same authors found that the under-vine grass/legume mixture favored a more valuable accumulation and resilience of both the SOC and the N in the rooting zone. However, it should be noted that both a higher grass root biomass and a higher C retention potential in the fine soil fractions due to the adsorption on minerals can explain the often observed increase in the fine SOC fraction (Giannetta et al., 2019b, 2018). Šimanský et al. (2023), in a long-term (14 years) vineyard trial, found that the SOC content increased in no-tillage/grass sward compared to no-tillage and 30 cm deep-ploughing. In the same trial, the labile organic carbon, i.e., the carbon that is readily decomposed by soil microorganisms and undergoes a rapid turnover (Haynes, 2005), did not show major changes over the years, although a higher trend, albeit highly variable, was observed for the no-till/turf option.

The principal active component of SOC is humus, which is formed by chemical processes in the natural environment (Zaiets and Poch, 2016). Humus can be fractionated into humin, fulvic acids (FA) and humic acids (HA). More specifically, HA and FA play crucial roles in soils, such as improving soil aggregate properties, promoting soil fertility, increasing soil buffering capacity, and regulating acidity and alkalinity (Kou et al., 2022; Nguyen et al., 2021). Some studies show that soil environmental disturbances (climate changes, agricultural fertilisation activity, type of soil management) can alter the structure and composition of HA and FA; the latter can then be used to assess the quality of soil management based on the degree of SOC humification (Canellas and Façanha, 2004; Machado et al., 2020; Raiesi, 2021; Sarkhot et al., 2007).

Microorganisms are also used as indicators of soil quality because they play key functions in the degradation and cycling of organic matter and nutrients (Dilly and Munch, 1998). Soil microbial biomass is the living component of the soil organic matter that includes organisms with volumes <5000 μm^3 and excludes macrofauna and roots. Microbial biomass is a good indicator of soil quality because it responds more rapidly than the total organic carbon to changes in soil conditions (Brookes, 2001). Although soil microbial biomass carbon (MBC) represents approximately 1 to 5 % (*w*/w) of the total organic carbon (Jenkinson and Ladd, 1981), variations in MBC content indicate changes in soil ecosystem functions and could imply potential deterioration. For example, in a recent study, Longa et al. (2017), while comparing the organic and biodynamic management of two vineyards in the Trentino region (Italy), found significant effects of green manure on soil microbiota, both in terms of soil biodiversity and on the relative abundance of specific bacteria and fungi involved in the nitrogen cycle. The effects of the different land management practices were pronounced not only on the bacterial community structure but also on the extracellular enzymatic activities of the soil. Indeed, the soil enzymatic activities could be a valuable indicator of microbial biomass activity and could provide important information on the effects of permanent grass cover, legume cover, and/or green manure on soil biological fertility (Cenini et al., 2016; Erdel and Şimşek, 2023; Trasar-Cepeda et al., 2008; Wallenius et al., 2011).

The soil enzyme activities involved in different nutrient cycles were analysed to provide an overall picture of the soil health status and to help compare potential differences between the management practices. The soil enzymes play a fundamental role in the biochemical processes of organic matter recycling and strongly influence soil physical properties, environmental quality, and agronomic productivity (Rao et al., 2014; Srinivasarao et al., 2018). Soil enzymes have been successfully used as ecological indicators because they provide early detection of changes and perturbations in the soil ecosystem, and they could be sensitive to changes in agronomic management practices and to the presence of xenobiotics (Lee et al., 2020; Shen et al., 2016).

The aim of this work is to identify the influence of two different soil management practices on the overall soil quality, providing essential ecosystem services. The conservation agriculture approaches are still a novelty in the viticulture sector, not widely adopted, and hardly considered by many producers. This experimentation hypothesised that different management techniques applied for the short and medium-term could have a distinctive impact on soil health.

For this reason, two different vineyard management practices, namely, an annual legume cover with Egyptian clover and a permanent meadow, were tested for four years on a farm in central Italy compared with an uncultivated soil used as control. The variations occurring in the different SOC forms and the activities of the soil microbial community were studied to evaluate the potential effects on both soil health and fertility after four years of experimentation.

The enrichment in SOC content was also tested for its resilience and stability, analysing the humic fraction, and the growth and activity of soil microbial biomass was tested to point out the capacity to mineralise the organic residues and sustain the carbon and nitrogen cycles.

Under the conditions of this "on-farm" experiment, the final aim of the work was to establish if four years of the two suggested conservative practices were sufficient to foster a significant SOC increase and a considerable shift towards more stable OC forms, improving both the chemical and biological fertility of vineyard soils.

2. Materials and methods

2.1. Site description

The study was conducted in a 9-year-old vineyard located in central Italy, in the countryside of Ancona (Maiolati Spontini; 43°27′36″N, 13°09′11″E). The vineyard was espalier with vines planted at 1,1 m row

Table 1
Summary of the main physico-chemical characteristics of the soil's samples from the three management options tested. Topsoil refers to the 0–20 cm layer and subsoil refers to the 20–40 cm layer. Data are the average of three replicates ± standard deviation.

Sample	pH (H_2O)	Texture	Sand (%)	Silt (%)	Clay (%)	Electrical Conductivity (dS m^{-1})	Cation Exchange Capacity (Meq 100 g^{-1})	Total N (g kg^{-1})	P available (g kg^{-1})
Annual legume cover topsoil	8.17 ± 0.08	Clay Loam	30.9	34.0	35.1	0.746 ± 0.01	22.01 ± 0.59	1.45 ± 0.03	12.11 ± 0.27
Annual legume cover subsoil	8.32 ± 0.08	Clay Loam	30.2	35.6	34.2	0.729 ± 0.02	21.12 ± 0.42	0.65 ± 0.02	3.76 ± 0.10
Permanent meadow topsoil	8.21 ± 0.06	Clay Loam	27.9	37.9	34.2	0.708 ± 0.01	21.06 ± 0.48	0.95 ± 0.03	6.17 ± 0.16
Permanent meadow subsoil	8.32 ± 0.04	Clay Loam	32.1	35.8	32.1	0.612 ± 0.01	20.03 ± 0.38	0.75 ± 0.02	2.60 ± 0.07
Control topsoil	8.21 ± 0.03	Clay Loam	33.3	34.3	32.4	1.068 ± 0.03	22.10 ± 0.55	0.85 ± 0.01	8.18 ± 0.18
Control subsoil	8.23 ± 0.05	Clay Loam	36.2	32.6	31.2	0.691 ± 0.01	21.31 ± 0.06	0.7 ± 0.02	7.10 ± 0.14

spacing and 3,0 m inter-row spacing, with a slope of 6–8 %; the grapevine cultivar was a "Verdicchio" grafted on 1103 Paulsen rootstock. According to the World Bioclimatic Classification System report by Rivas-Martìnez (1993), the vineyard belongs to the temperate macroarea, more specifically, the sub-mediterranean bioclimatic variant (Pesaresi et al., 2017). The soil was classified as Haplic Calcisol according to the database of Italian Typological Units (ASSAM, 2006).

The vineyard was selected among different organic fields available in the same region that were tested according to homogeneous soil characteristics in terms of organic matter content.

Two different inter-row management practices were evaluated in this vineyard, respectively: annual legume cover (total area of 13,900 m^2) and permanent meadow cover (total area of 12,900 m^2). The length of the rows varied from a minimum of 100 m to a maximum of 150 m, and the areas of the two treatments were adjacent.

In the annual legume cover area, the cover was realised for four consecutive years with Egyptian clover (*Trifolium alexandrinum* L.) sown at 30 kg ha^{-1} using the sod seeding technique, sown in early autumn for two years, in late summer for one year, and in spring for one year due to climatic constraints. The crop was mowed three or two times per year, leaving the crop residues on the site as mulch, i.e. without involving any tillage. The plant density ranged between 600 and 700 plants per m^2, and the soil cover percentage was close to 100 % for the four years. Likewise, in the permanent meadow area, the permanent grassland was achieved for four years by spontaneous species and reached a cover percentage close to 95 %, mowed three or two times per year and mulched without any tillage. The species identified in the permanent meadow were mainly *Poaceae,* with a major presence of oats (*Avena* spp.) and a minor presence of both couch grass (*Elymus repens* L. Gould) and ryegrass (*Lolium perenne* L.). Frequently, there were identified *Polygonaceae* such as docks (*Rumex* spp.), *Asteraceae* such as crepis (*Crepis* spp.) and dandelions (*Taraxacum* spp.), *Plantaginaceae* like the ribwort plantain (*Plantago lanceolata* L.), and in smaller quantity *Fabaceae* such as white clover (*Trifolium repens* L.) and spotted medick (*Medicago arabica* L. Huds.).

In addition, a soil area of 1374 m^2 adjacent to the two experimental inter-rows that had not been cultivated for 20 years was used as a control; this control was selected since it was representative of the same soil but with long-term, stable and steady SOC dynamics and it would have provided more useful information than a conventional vineyard, indeed not available in the nearby areas. This area was mowed and mulched as the other two treatments, but due to the rare presence of spontaneous species during the year, a mean soil cover of 30 % was observed.

Organic fertilisation was applied to the whole vineyard every year in autumn, distributing 0.15 t ha^{-1} of cattle manure in the inter-row, equivalent to 80 nitrogen units ha^{-1}.

The sampling was done in late fall (November). Following the recommendations of Italian handbook for chemical soil analyses (Violante, 2000) five sampling points were selected for each thesis, and two soil samples were collected for each sampling point at two depths, namely 0–20 cm (topsoil) and 20–40 cm (subsoil). The five sampling points were determined according to a systematic grid sampling method based on homogeneous areas for both the treatment annual legume cover and permanent meadow, while for the control soil area, due to its different shape and size, the sampling points were equidistantly set, as shown in Fig. S1 of the supplementary materials. A preliminary soil sampling according to the same procedure was also performed before the trial onset to evaluate the uniformity of the soil characteristics under scrutiny; these data are reported in the supplementary information (Table S1).

Each sample was hand-crushed and sieved 2 mm to obtain approximately 3 kg of fine earth. The soil samples obtained from each of the five sampling points were thoroughly mixed in equal amounts and homogenised to obtain one macro-sample per treatment type and depth; macro-samples were stored at 4 °C. This procedure allowed obtaining mean values of each chemical and biochemical properties taking into account the spatial variability within the treatments and the analytical errors, as often suggested for this type of determinations. This sampling protocol was adopted since the preliminary analysis performed showed that the differences between the vineyard rows for the organic carbon content were negligible and statistically non-significant.

Depending on the type of analysis, the soil was air-dried or analysed at field moisture level. Three sub-samples from each macro-sample were taken for organic carbon analyses, while five for enzymatic activity determinations. The main soil characteristics of each macro sample are shown in Table 1; the analyses were carried out from each macro-sample, according to the standard protocols published in the Italian Official Gazette n°248 (Adamo and Violante, 2000). In Table S2 of supplementary material, the concentrations of some metals and other elements of interest are reported.

2.2. Assays

The soil quality in the areas treated with different land management was assessed considering chemical and biochemical soil parameters. The chemical tests performed were mainly related to the content of organic matter and the distribution of different forms of organic carbon. Organic matter positively influences several soil properties and plays a fundamental role in forming and maintaining its physical and biological structure (Ciavatta et al., 1989). Furthermore, biochemical tests were carried out to examine the microbial biomass of the soil and the enzymatic activities to investigate the biological effects of the two different management practices.

2.2.1. Chemical analyses

Total organic carbon content (TOC) and total extractable carbon (TEC) are key parameters in soil quality assessment. The total organic carbon was determined according to the standard method (WB) developed by Walkley and Black (1934); the organic matter is oxidised with potassium dichromate in the presence of sulfuric acid. The amount of unreacted potassium dichromate is quantified by titration with ferrous ammonium sulphate.

TEC, humic acids (HA), and fulvic acids (FA) were measured by three separate titrations, according to the method proposed by Schnitzer (1982). Part of the extracted TEC was used for further fractionation to extract HA and FA; the HA were collected after precipitation with sulfuric acid, while the FA was adsorbed on a polyvinylpyrrolidone (PVP) insoluble matrix. Finally, the carbon content in each fraction was determined using the WB method, as reported previously by other authors (Vignozzi et al., 2023; Vischetti et al., 2020).

The fractionation results obtained were used to calculate the non-humified fraction (NH), the humification index (HI), the humification degree (DH) and the humic/fulvic acids ratio (CHA/CFA) according to the following formulas:

$$\mathrm{NH} = \mathrm{TEC} - (\mathrm{HA} + \mathrm{FA})\ \left[\mathrm{g\ kg}^{-1}\right]$$

$$\mathrm{HI} = \mathrm{NH}/(\mathrm{HA} + \mathrm{FA})$$

$$\mathrm{DH} = (\mathrm{HA} + \mathrm{FA})/\mathrm{TEC}\ [\%]$$

$$\mathrm{CHA/CFAHA/FA}$$

The HI index reaches values close to zero for a completely humified TEC; the lower the HI value, the more the humification processes predominate over the mineralisation processes. The DH provides information on the percentage of humic substances compared to the TEC; high DH values indicate a greater ability of the soil to humify the available organic matter (Monaci et al., 2017; Sanesi et al., 2017). The CHA/CFA ratio indicates good humus quality when the ratio is greater than one since the HA fraction is mainly responsible for soil fertility and could promote microbial activity (Becher et al., 2020).

2.2.2. Microbiological analyses

Microorganisms are also used as indicators of soil quality because they play key functions in the degradation and cycling of organic matter and nutrients (Dilly and Munch, 1998). Soil microbial biomass is the living component of the soil organic matter that includes organisms with volumes $<5000\ \mu\mathrm{m}^3$ and excludes macrofauna and roots. Microbial biomass is a good indicator of soil quality because it responds more rapidly than the total organic carbon to changes in soil conditions (Brookes, 2001). Although microbial biomass carbon (MBC) represents approximately 1 to 5 % (*w*/w) of the total organic carbon (Jenkinson and Ladd, 1981), variations in microbial biomass content indicate changes in soil ecosystem functions and could imply potential deterioration.

The MBC was estimated using the fumigation-extraction method, and it was calculated as the difference between the amount of C present in sample aliquots fumigated with chloroform and the C in the corresponding non-fumigated samples (Vance et al., 1987).

Another estimated indicator of soil biological fertility was microbial respiration, which measures the activity of the microbial biomass present in the soil samples. The microbial activity was monitored by applying the method developed by Dumontet and Mathur (1989); the microorganisms obtain energy from organic compounds, and their metabolic activity can be quantified by measuring carbon dioxide production. The microbial activity was monitored by tracking the cumulative CO_2 produced by samples over approximately 45 days until a production plateau was reached. The CO_2 from the samples was captured by NaOH traps and then estimated by titration every 5 days. The cumulative respiration (Ccum) was expressed as mg CO_2–C kg^{-1} soil. On the last day of incubation, the basal respiration (bSR), i.e. the respiration rate of the initial microbial community under steady-state conditions, was also recorded and measured as mg CO_2–C kg^{-1} soil d^{-1}. These results were used to calculate both the metabolic quotient (qCO_2) and the mineralisation quotient or normalised soil respiration (nSR) according to recent publications (Alfaro-Leranoz et al., 2023; Pereira et al., 2023) and the formulas were reported as following:

$$\mathrm{qCO_2} = \mathrm{bSR}\ (\text{basal respiration})/\mathrm{MBC}\ (\text{microbial carbon biomass})$$

$$\mathrm{nSR} = \mathrm{Ccum}\ (\text{cumulative respiration})/\mathrm{TOC}\ [\%]$$

The metabolic quotient qCO_2 is the CO_2 emitted per hour per unit of microbial biomass (Anderson and Domsch, 1993); it expresses the relationship between the microbial activity and the amount of microbial biomass, and it allows estimating the effects of external disturbances. The nSR quotient indicates the efficiency of the micro-flora in metabolising SOC (Dommergues, 1960).

2.2.3. Enzymatic activity analyses

The fluorescein diacetate hydrolytic activity (FDA), the alkaline phosphatase, the β-glucosidases, the ortho-diphenol oxidase, the β-*N*-acetyl-glucosaminidase (NAG), and the leucine aminopeptidase (LAP) assays were performed on all soil samples. All the enzymatic analyses were performed using spectrophotometric methods.

The FDA enzymatic assay summarises the hydrolytic activity of numerous enzymes, such as proteases, lipases, and esterases; it measures the total potential degrading activity of fungi and bacteria (Green et al., 2006). The amount of fluorescein diacetate hydrolysed in 1 h was estimated using Schnurer and Rosswall's (1982) method.

Alkaline phosphatase is an indicator of the potential for organic P mineralisation and P availability in the soil (Tabatabai, 1994). The phosphatase assay includes a large group of enzymes that catalyse the hydrolysis of phosphate esters (Nannipieri et al., 2011). The analysis was performed using the Eivazi and Tabatabai (1977) method, which quantifies the p-nitrophenol released in 1 h from a *p*-nitrophenyl phosphate substrate added to soil samples.

The β-glucosidases are extracellular enzymes belonging to the class of hydrolases that catalyse the hydrolysis of oligosaccharides. They are involved in the C cycle and are positively associated with soil organic matter (Ferraz De Almeida et al., 2015; Luo et al., 2017). The method of Eivazi and Tabatabai (1988) was adopted; the amounts of p-nitrophenol released in 1 h from a *p*-nitrophenyl-β-D-glucopyranoside substrate added to the soil's samples were estimated.

Ortho-diphenol oxidase is another enzyme involved in the C cycle; it is an indicator of the efficiency of the conversion of polyphenols from organic matter to humus and is mainly related to fungal activity. The spectrophotometric method developed by Perucci et al. (2000) determines a red compound (4-(N-proline)-o-benzoquinone) formed in soil samples after the oxidation of a catechol substrate in the presence of proline.

The β-*N*-acetyl-glucosaminidase (NAG) is a hydrolytic enzyme that hydrolyses oligosaccharides, including chitin and peptidoglycan, and is involved in the N and C cycles (Ekenler and Tabatabai, 2002). The method of Parham and Deng (2000) quantifies the p-nitrophenol released in 1 h from a *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminidine substrate added to the soil's samples.

Leucine aminopeptidase (LAP) is a soil hydrolytic enzyme involved in the nitrogen cycle; microorganisms use this enzyme to catalyse the hydrolysis of polypeptides to promote nitrogen acquisition. For the LAP assay, a substrate solution of leucine p-nitroanilide was used following the method suggested by Yan et al. (2020).

2.3. Statistical analysis

Statistical analyses were performed in R software (R Core Team, 2022, version 2022.06.0 + 421) using ANOVA when the data met the assumptions, considering overall six factors as a combination of the

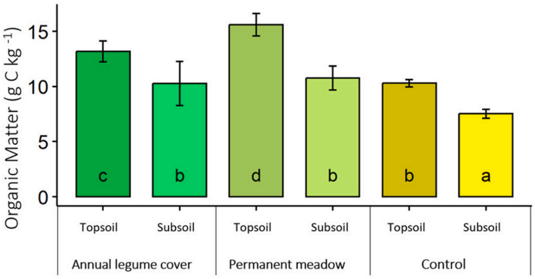

Fig. 1. Organic matter content in soil samples (n = 3). Topsoil refers to the 0–20 cm layer and subsoil refers to the 20–40 cm layer. Different letters indicate statistically significant differences according to ANOVA and Tukey post-hoc multiple comparisons test (α-level = 0.05).

three theses and the two depths. Where the assumptions were not met, the non-parametric Kruskal-Wallis's and Dunn's post hoc tests were used (Bonferroni *p-value* adjustment, α = 0.05). Three or five replicates were performed per determination, as described above in the sampling procedure.

3. Results and discussion

In the following sections are summarised and discussed the overall effects of the two conservative practices on the organic matter content and on soil enzymes related to carbon, phosphorus and nitrogen cycles.

The discussion was developed to highlight the significant differences found between the two conservative practices and the soil control at both the depth tested and for all the parameters determined. The results could clarify if four years of conservative vineyard management can lead to an enrichment in stable and resilient forms of organic carbon and to an increase in the growth and activity of soil microbial biomass.

3.1. Organic matter content

The organic matter content (OMC) of all three theses in both the topsoil and subsoil is shown in Fig. 1. OMC data were calculated from the TOC based on the generic conversion (OMC = TOC x 1.72), assuming a stoichiometric average of 58 % C in soil organic matter (Pribyl, 2010).

As can be seen, there are significant differences between the two management options in the topsoil layer: the soil with the highest OMC concentration was the permanent meadow (15.6 g Kg^{-1} on average), followed by the annual legume cover (13.2 g Kg^{-1}), and the control (10.3 g Kg^{-1}). The first two values could be considered typical values for Italian vineyard topsoil, being values in the range of 12 to 20 g Kg^{-1} (Castaldi, 2014), while the third is a low value classifiable in the range of 8 to 12 g Kg^{-1}, indicating the good performance of the two conservative practices compared to the control. Although these results are promising and show a clear improvement, it should be stressed that these soils still need many more years with these conservative practices to reach the acceptable value of 2 %, a minimum concentration indicated for organic vineyards in Italy (Morelli et al., 2022) and more in general in Europe (Jaksic et al., 2021).

In the 20–40 cm subsoil layer, the two soil management systems did not show any significant differences in the OMC, although both were significantly higher than the control, probably due to the higher presence of root residues.

The increase in organic carbon content following conservative practices such as no-tillage and/or minimum tillage combined with green manure or permanent grass cover has been reported in several agronomic studies (Angeletti et al., 2021; Gao et al., 2018; Saviozzi et al., 2001), although in fewer and more recent cases in vineyards (Ball et al., 2020; Jaksic et al., 2021; Šimanský et al., 2023). In these latter studies, the introduction of permanent grass cover, mulching, and/or green manure often increases organic carbon, especially in the topsoil. However, this does not automatically imply that soil fertility could increase permanently when green residues are added.

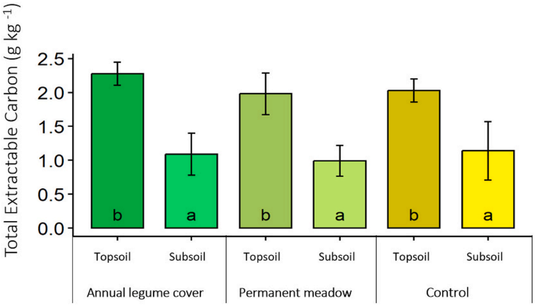

Fig. 2. Total extractable carbon (TEC) in soil samples (*n* = 3). Topsoil refers to the 0–20 cm layer and subsoil refers to 20–40 cm layer. Different letters indicate statistically significant differences according to ANOVA and Tukey post-hoc multiple comparisons test (α-level = 0.05).

3.2. Total extractable carbon

In order to better understand whether the OC supplied remained stable over time, it was necessary to study the different forms of OC derived from these conservative practices, i.e., whether a significant proportion of OC was converted into a "stable and resilient" humus. Depending on the nature of the organic residues, the pedo-climatic conditions, and the microbial growth and activity, soil OC can undergo a series of chemical and biological processes that could degrade it faster or slower, transforming it into stable organic carbon compounds such as humus (Monaci et al., 2017). Moreover, it could also be protected in soil aggregates and/or organo-mineral fractions (Angeletti et al., 2021; Giannetta et al., 2019b).

The amounts of total extractable carbon (TEC) were analysed in all soil samples and the results are shown in Fig. 2.

The percentage of TEC to TOC, calculated in the topsoil, ranged from 21.8 % in the permanent meadow to 33.9 % in the control (Table 2). The TEC content was similar in the three theses, showing no effect of the conservation practices on this OC form. The content in the topsoil was significantly higher than that in the deeper soils in all samples, indicating that this form of OC is mainly located where grassroots and microbial activity are more intense (Canellas et al., 2004; Orlov, 1998).

Francaviglia et al. (2017) found a TEC to TOC ratio varying from 71 to 81 % in topsoil of different managements, such as tilled and untilled grassed vineyards, indicating a low presence of humin, i.e., the OC fraction bound to the soil mineral components. On the contrary, in another experiment, Saviozzi et al. (2001) found low values, around 18 %, in grassy and forest topsoil. This implies that a remarkable fraction of the OC was bound in organo-mineral soil components and then protected from degradation and release into the atmosphere as CO_2.

On the other hand, the role of mineral fraction in the capture and stabilisation of organic carbon has been demonstrated by several reports (Giannetta et al., 2019a, 2018).

3.3. Humic and fulvic acid fractions

Figs. 3 and 4 show the humic (HA) and fulvic (FA) acid fractions obtained from further separations of all TEC samples. The HA content in the topsoil of the annual legume cover treatment resulted significantly higher than the other two theses, while in the deeper soil, it showed a HA content significantly lower than the other two, indicating that the humification process in the topsoil of the legume cover crop management proceeded well. This is in agreement with the results found from similar experiments in vineyards (Morelli et al., 2022; Šimanský et al., 2023), showing that green manure practices increase organic carbon content in the topsoil and generate forms of carbon resistant to microbial activity.

Table 2

Organic carbon indices of the soil samples (means ± SD). Topsoil refers to the 0–20 cm layer and subsoil refers to the 20–40 cm layer. Total Extractable C (TEC) to Total Organic Carbon (TOC) percentage, Non Humified C fraction (NH), Humification Index (HI), Degree of Humification (DH), and Humic Acid/fulvic C ratio (CHA/CFA). Different letters indicate statistically significant differences according to ANOVA and Tukey post-hoc multiple comparisons test (α-level = 0.05).

Sample	TEC/TOC (%)	NH (g kg^{-1})	HI	DH (%)	CHA/CFA
Annual legume cover topsoil	29.82 ± 0.10c	1.23 ± 0.14b	1.17 ± 0.11b	46.19 ± 2.41 ac	2.16 ± 0.51b
Annual legume cover subsoil	18.26 ± 4.41ab	0.76 ± 0.25ab	2.29 ± 0.45b	30.80 ± 4.56a	0.51 ± 0.31a
Permanent meadow topsoil	21.83 ± 3.41 ac	1.29 ± 0.17b	1.95 ± 0.51b	34.62 ± 6.27a	1.09 ± 0.10b
Permanent meadow subsoil	15.78 ± 2.75a	0.42 ± 0.29a	0.77 ± 0.56a	58.00 ± 9.91bc	2.67 ± 0.31 b
Control topsoil	25.69 ± 2.90bc	1.23 ± 0.17b	1.53 ± 0.21b	39.69 ± 3.20ab	0.78 ± 0.14a
Control subsoil	16.16 ± 5.95ab	0.45 ± 0.47a	0.67 ± 0.72a	60.87 ± 10.75c	0.90 ± 0.09a

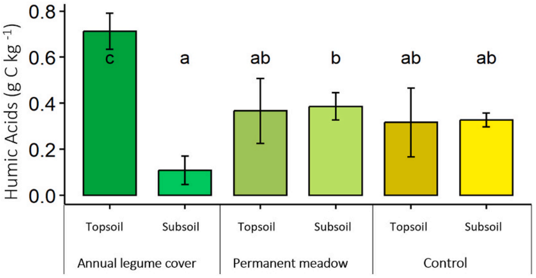

Fig. 3. Humic acid carbon (HA) in soil samples (n = 3). Topsoil refers to the 0–20 cm layer and subsoil refers to the 20–40 cm layer. Different letters indicate statistically significant differences according to ANOVA and Tukey post-hoc multiple comparisons test (α-level = 0.05).

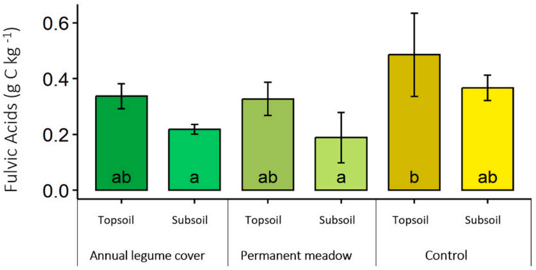

Fig. 4. Fulvic acid carbon (FA) in soil samples (n = 3). Topsoil refers to the 0–20 cm layer and subsoil refers to the 20–40 cm layer. Different letters indicate statistically significant differences according to ANOVA and Tukey post-hoc multiple comparisons test (α-level = 0.05).

However, in other experimental conditions, especially in conventional cropland, grass cover and/or green manure result in low values of TEC and CHA/CFA plus HA, similar to conventionally cultivated soils (Monaci et al., 2017; Saviozzi et al., 2001).

The FA data, presented in Fig. 4, did not show any significant differences between the management options, except for the control, where the slightly higher values for the fulvic acids seem to indicate a lower quality of the humified fraction. These results confirm that the conservative practices adopted could effectively contribute to quantitatively and qualitatively increasing the SOC.

3.4. Organic carbon indices

Table 2 shows the most relevant OC indices determined for the two soil management practices. The estimated values of the humification index (HI) indicated a low humified TEC, especially in the three topsoils; this was expected since organic fresh residues are often more predominant on topsoils. The degree of humification (DH) was calculated as 46.19 % ± 2.41, 34.62 % ± 6.27, and 36.69 % ± 3.20 in the topsoil of the annual legume cover, permanent meadow and the control, respectively. In the subsoil, the DH values in the permanent meadow option and the control were significantly higher than in the legume cover; one of the reasons may be that the subsoil of these two theses remained undisturbed for many years, allowing for greater humus production.

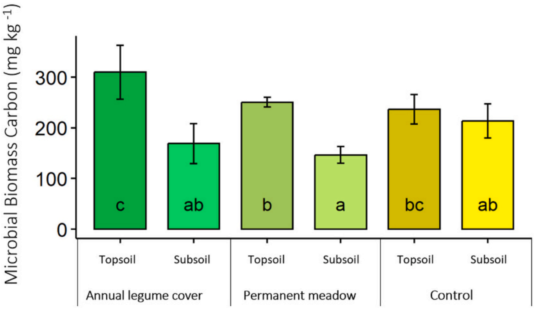

Fig. 5. Microbial-C in soil samples (n = 3). Topsoil refers to the 0–20 cm layer and subsoil refers to the 20–40 cm layer. Different letters indicate statistically significant differences according to ANOVA and Tukey post-hoc multiple comparisons test (α-level = 0.05).

The HI values observed here were higher than those reported in other experiments on grassy soils (Saviozzi et al., 2001) and vineyard grassy soils (Francaviglia et al., 2017) with a long history of conservation practices. In fact, as expected, the DH values obtained in these long-term trials were higher than in our tests since more time is usually needed to reach a higher degree of humification. On the other hand, even if the humification is in an intermediate state, the quality of the humus can be considered satisfactory, looking at the values of the CHA/CFA ratio. This ratio is very informative and particularly important due to the multiple functions that humic acids exert on the chemical and biological fertility of the soil (Mbarek et al., 2020); this ratio should always be higher than one to ensure that the humification process proceeds in an optimal way (Angelova et al., 2013; Kononova, 2013). In our trial, even if the DH showed low values, the CHA/CFA ratio resulted in high values both in the permanent meadow and in the topsoil covered with legumes, indicating both the presence of good-quality humus, resistant to the attack of microbial biomass and the preservation of OC from excessive mineralisation.

3.5. Microbial biomass growth and activity

3.5.1. Biomass carbon content

The results of the microbial biomass carbon (MBC) content analysis in the two soil management practices are summarised in Fig. 5. The annual legume cover showed a topsoil value significantly higher than that of the permanent meadow and that of the control, indicating that this kind of conservative practice strongly contributes to increase the microbial biomass growth in the topsoil. The values of the subsoils for the three theses didn't show significant differences between them, and the result was lower than that of the topsoils. However, these conservative practices often do not contribute to increase the total amount of microbial biomass, also in the topsoil, as observed by other authors (Monaci et al., 2017; Saviozzi et al., 2001) for permanent grasslands,

Table 3

Averages of selected parameters related to the soil's microbial activity (means ± SD). Topsoil layer: 0–20 cm; subsoil layer:20–40 cm. Microbial biomass carbon (MBC); basal soil respiration (bSR); soil cumulated respiration (Ccum); metabolic quotient (qCO2); normalised soil respiration (nSR); soil Carbon/Nitrogen ratio (C/N). Different letters indicate statistically significant differences according to ANOVA and Tukey post-hoc multiple comparisons test (α-level = 0.05).

Sample	MBC (mg kg^{-1}soil)	bSR (mg CO2-C kg^{-1}soil d^{-1})	Ccum (mg CO2-C kg^{-1}soil)	qCO2 (mg CO2-C h^{-1}mg MBC^{-1})	nSR (%)	C/N
Annual legume cover topsoil	310.01 ± 53.30c	9.05 ± 1.90c	700.7 ± 41.1d	1.26 ± 0.52c	9.18 ± 0.16c	5.27 ± 0.38a
Annual legume cover subsoil	168.98 ± 39.19ab	2.76 ± 0.62b	379.3 ± 10.7b	0.73 ± 0.36b	6.29 ± 1.24b	9.17 ± 1.76b
Permanent meadow topsoil	250.88 ± 9.21bc	5.24 ± 0.34b	448.7 ± 6.11c	0.87 ± 0,10ab	4.97 ± 0.36ab	9.53 ± 0.58b
Permanent meadow subsoil	146.69 ± 16.48a	3.05 ± 0.32b	267.3 ± 6.01a	0.88 ± 0.22a	4.32 ± 0.50a	8.32 ± 0.84b
Control Topsoil	236.83 ± 28.97 ac	2.19 ± 0.39a	352.0 ± 8.32b	0.39 ± 0.13a	4.48 ± 0.67a	9.38 ± 1.33b
Control Subsoil	213.72 ± 33.93ab	2.67 ± 1.05a	358.0 ± 16.2b	0.55 ± 0.33ab	5.10 ± 0.40ab	10.05 ± 0.33b

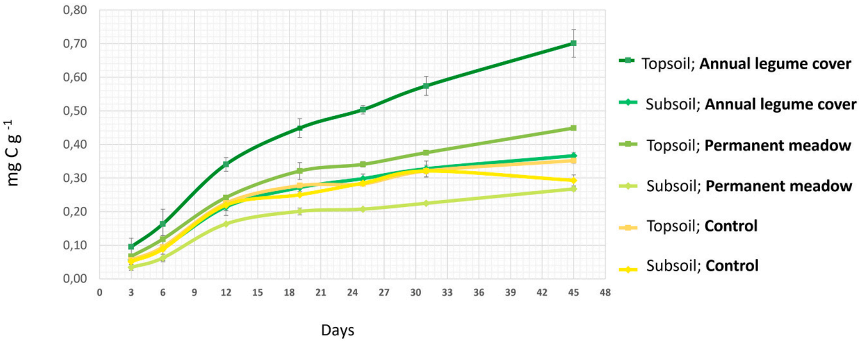

Fig. 6. Cumulative respiration of soil samples (n = 3).

cover crops, or green manures.

3.5.2. Soil respiration

Both the basal and cumulative soil respiration were estimated for all the thesis studied and the results are reported respectively in Table 3 for the basal, and in Fig. 6 for the cumulative respiration. The basal respiration was significantly higher in the annual legume cover soil, followed by the permanent meadow and the control. The two cultivated soils showed large differences between the two depths, also related to the differences in microbial C content (Fig. 5).

The cumulative respiration observed in the three soils at two depths is shown in Fig. 6, while the relative statistical analysis is reported in table S3 of the supplementary material. Overall, the respiration was significantly higher in the annual legume cover than in the permanent meadow and in the control soil, with differences between the two depths, as well as in the permanent meadow soil, although at a lower rate. The lowest respiration was recorded in the control, with no significant differences between the two depths.

The basal and cumulative (bSR, Ccum) respiration data were also used to calculate the metabolic quotient (qCO_2) and the normalised soil respiration (nSR). All these parameters correlated with the soil microbial activity at the two depths of the different management practices are summarised in Table 3.

The derived qCO_2 values were mostly lower than one, except for the annual legume cover soil at 0–20 cm depth, which is close to one (1.26), indicating no specific disturbance in the activity of the microbial biomass which shows a very low level of stress. Other studies on soils managed with different grasses or manures and with different land uses reported values comparable to those found in the present experiment (Francaviglia et al., 2017; Saviozzi et al., 2001; Yan et al., 2003). The high values of qCO_2 were often associated with a high C/N ratio in the soil litter layer, with a positive correlation between soil basal respiration and the C/N ratio and a positive correlation between qCO_2 and soil N concentration (Antisari et al., 2021; Spohn, 2015). The present work seems to partially confirm these findings, showing that for the annual legume cover topsoil both a high value of basal respiration and qCO_2, although with a high N concentration and a low C/N ratio. However, apart from this case, the other results did not show significant differences between the different soils' management in terms of the C/N ratio except for the topsoil in the annual legume cover management. In fact, a C/N ratio between 1 and 15 indicates an optimal mineralisation of organic matter by the soil microbial community and the consequent release of N into the soil for immediate use by plants (Brust, 2019; Watson et al., 2002). It is important to note that the basal respiration of the annual legume cover topsoil was relatively higher, indicating a higher level of mineralisation and CO_2 release. This should be considered when a legume, such as *Trifolium alexandrinum* L., is used as a cover crop. If a cover crop and/or green manure are programmed for vineyard soil, it may be more appropriate to sow a mixture of leguminous and non-leguminous seeds. Similarly, the nSR values were high in all the theses and particularly high in the topsoil of the annual legume cover. These data indicate a very active microbial community that is efficient in metabolising SOC, which is in agreement with information reported in the literature for both uncultivated and vineyard soils (Pinzari et al., 1999; Sharma et al., 2020; Yaghoubi Khanghahi et al., 2019).

3.5.3. Soil enzyme activities

The activities of the FDA hydrolysis, the β-glucosidase, the orthodiphenoloxidase, and the alkaline phosphatase are shown in detail in Fig. 7.

For the FDA hydrolytic activity, no significant differences were found between the topsoil of the three theses, while significant differences were found in the subsoil, where the highest activity was observed in the annual legume cover soil, followed by the permanent meadow (Fig. 7a). Being the FDA the sum of all the hydrolytic enzymes, it was expectable that the three theses didn't differ in the topsoil, where plant residues were present in a noticeable amount. The differences found in the subsoil could be ascribed to the different management of the three areas under study; in the annual legume cover and permanent meadow the high density of roots and residues also in the subsoil, especially in the

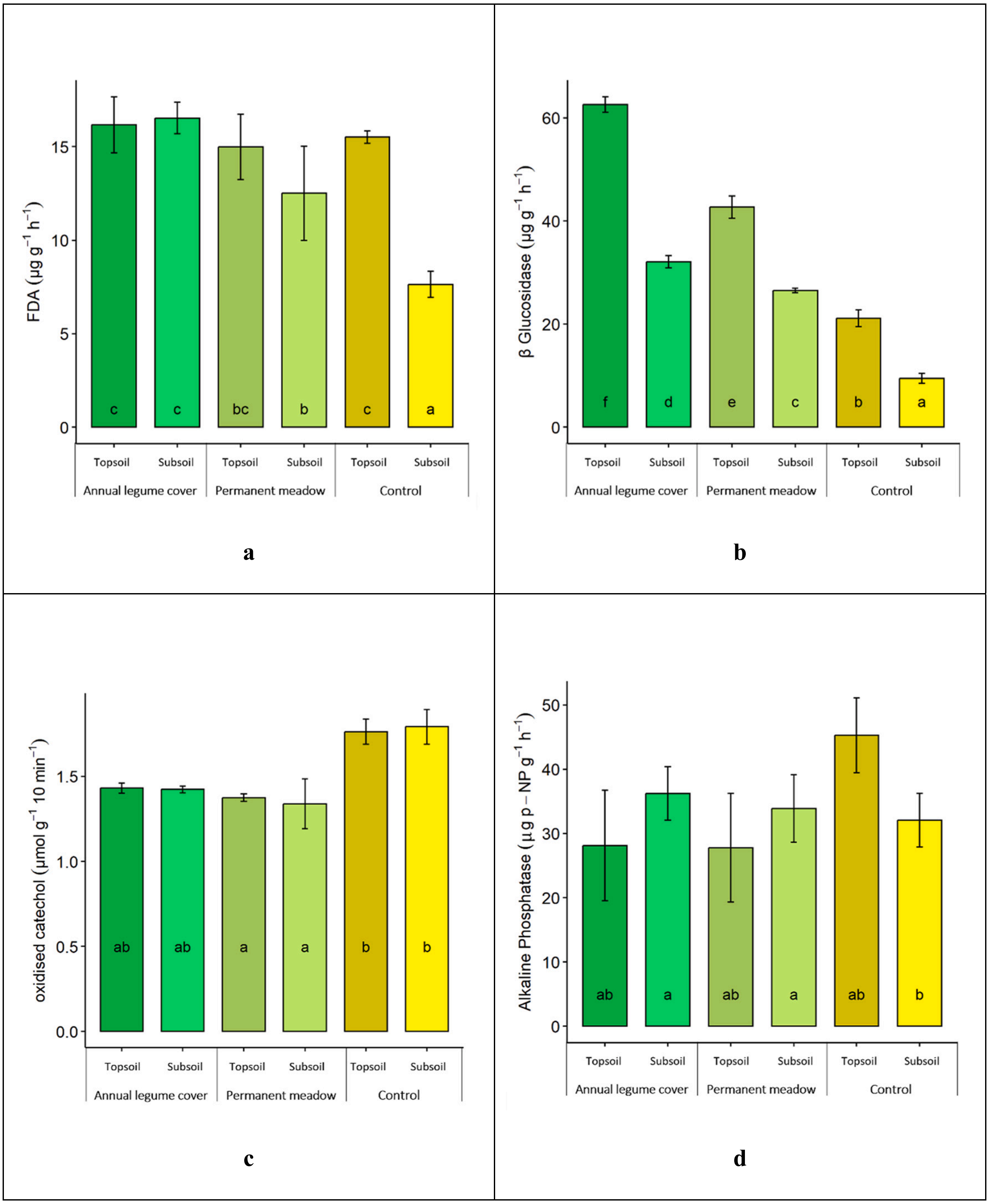

Fig. 7. Average ($n = 5$) soil enzyme activities linked to carbon and phosphorus cycles. FDA hydrolysis (a); β-glucosidase (b); ortodiphenoloxidase (c); alkaline phosphatase (d). Different letters indicate statistically significant differences according to Tukey post-hoc multiple comparisons test (α-level = 0.05) for FDA and β-glucosidase and according to Kruskal–Wallis multiple comparisons (Benjamini–Hochberg p-value adjustment, α-level = 0.05) for ortodiphenoloxidase and alkaline phosphatase.

first, give FDA values higher than control soil, where no tillage was performed during the last 20 years and the plant residues were rare. Anyway, a strong relationship was observed between SOM content and/or microbial biomass content and hydrolytic enzyme activity (Trasar-Cepeda et al., 2008; Wilkerson and Olapade, 2020).

Regarding the β-glucosidase activity, significant differences were found between the theses, with the highest values in the annual legume cover topsoil and a large reduction, about two-thirds and one-third, respectively, for the permanent meadow and the control; the same trend was followed in the subsoils (Fig. 7b). These results indicate that the microbial biomass present in the annual legume cover soil is much more active in degrading cellulosic residues and explains the production of a humified fraction higher than the other two treatments. Similar results were found by other authors who stated that green manures, cover crops, or permanent grasses increase the β-glucosidase activity in soils (Adetunji et al., 2020; Cenini et al., 2016; Saviozzi et al., 2001; Wallenius et al., 2011).

The orthodiphenoloxidase activity was significantly higher in the control area, indicating a higher complexity of the organic compounds' structures involved in the humification process under these conditions

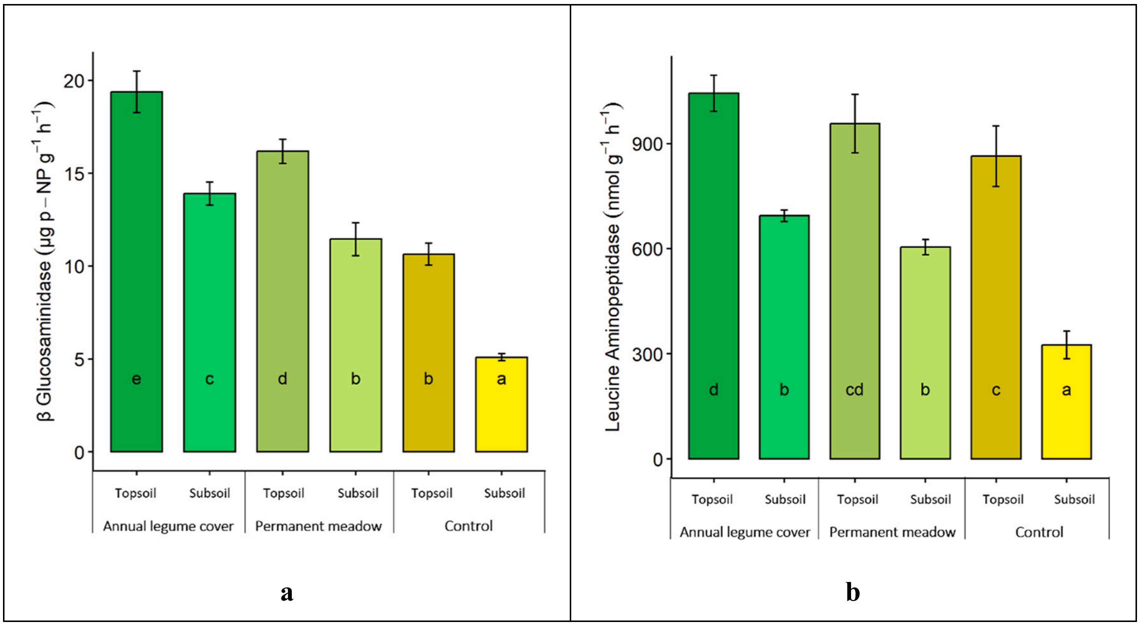

Fig. 8. Average (n = 5) soil enzyme activities. (a) β-1,4-*N*-acetylglucosaminidase (NAG); (b) Leucine Aminopeptidase (LAP).

(Fig. 3c). This is partially consistent with the data reported in Figs. 3 and 4, where the control had a high FA content with a C_{HA}/C_{FA} ratio of 0.65 in the topsoil and 0.89 in the subsoil, suggesting a sustained ortodiphenoloxidase activity to transform the FA fraction.

The alkaline phosphatase activity did not show any differences between the three soils at the topsoil level, and it seems that the two conservative practices did not increase this activity; no differences among the three soils are probably due to an already good, relatively higher enzyme activity in the control rather than a lower activity of the other two soils' management. The values found in the present experiment for the control soil are higher than those reported by Wallenius et al. (2011) for different soil managements (Erdel and Şimşek, 2023; Wallenius et al., 2011). In addition, the β-1,4-*N*-acetylglucosaminidase (NAG) and leucine aminopeptidase (LAP) were measured in this experiment because of their key role in soil organic matter decomposition and nutrient cycling. The results of the activities of these two soil enzymes involved in C and N cycling are shown in Fig. 8.

The NAG activity was significantly high in the annual legume cover treatment, especially at the topsoil level, indicating a good mineralisation capacity for this soil; the permanent meadow also showed the NAG activity significantly higher than the control. It should also be noted that the soil N concentration in the annual legume cover was high in both the top and bottom soil layers, certainly due to the nitrogen fixation capacity of the Egyptian clover (Table 3). It is widely recognised that the immediate availability of soil N can reduce NAG and LAP activity (Qu et al., 2021; Saiya-Cork et al., 2002; Uwituze et al., 2022; Zhang et al., 2016), but this is to be expected when inorganic N is supplied as soil N enrichment and soil microorganisms are induced to utilise this form of N. On the contrary, when N is supplied with organic fertilisation, the hydrolytic activity of microorganisms could be stimulated, and thus NAG and LAP activity could increase. This is probably what happened in the present study, where a large organic supply occurred.

4. Conclusions

The present paper highlights the importance of conservative practices such as annual legume cover and permanent meadow in vineyard soil management. In the conditions of the present experiment, an overall increase in organic carbon concentration was found in the 40 cm soil layer compared to the uncultivated soil. The annual legume cover soil showed a content of humic acids in the topsoil significantly higher than the permanent meadow and the control, indicating that this practice could contribute to a stable and resilient SOC stock. The topsoil MBC of the annual legume cover, as well as the soil respiration, was significantly higher than that of the permanent meadow. In the annual legume cover soil, there was a higher degradation of cellulose residues and, in general, higher basal respiration, indicating a lower efficiency in terms of C balance. The activity of enzymes involved in the N cycle was correlated with soil management, showing a better performance in the cover crop option, especially in the annual legume cover management. These results suggest that, in on-farm conditions similar to the condition of this trial, i.e. vineyard soils under an organic management, the cover crop option should be considered instead of the more conventional no-tillage. The beneficial management options proposed here could help to improve both soil health and fertility. The work could help to develop more effective strategies to reduce the impact of agricultural activities on vineyard soil ecosystems, such as traditional weed management, and to improve both SOC storage and nutrient cycling. These four years results represent preliminary observations on the SOC mutations that often occur very slowly in medium to long term periods. Long term trials with multiple years intervals, and in multiple sites, would be beneficial to test, respectively, measurable modifications on soil health and fertility, and to be able to generalize these results for large viticultural areals.

CRediT authorship contribution statement

Arianna De Bernardi: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Enrica Marini:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Francesca Tagliabue:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Gianluca Brunetti:** Writing – review & editing, Writing – original draft, Visualization, Validation. **Cristiano Casucci:** Writing – review & editing, Validation, Methodology, Data curation. **Überson Boaretto Rossa:** Writing – review & editing, Investigation, Formal analysis. **Oriana Silvestroni:** Writing – review & editing, Supervision, Project administration. **Costantino Vischetti:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2025.105868.

Data availability

Data will be made available on request.

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