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“Seasonal variation of enzyme activity in fields under different management practices”

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Abstract

The intensified conventional agriculture practices have as an effect the deterioration of soil health, threatening the resilience of agroecosystems to climate change. The adverse effects of climate change on soil health are especially alarming in arid and semi-arid regions like the Mediterranean basin, where inputs of organic matter are already low due to limitations imposed by high temperature and water scarcity. Decline in soil organic matter, along with decrease of fertility and biodiversity loss are among the impacts on soil health. Soil organic matter is inextricably linked with soil health and fertility, serving as the main energy source for soil microbiota and playing a crucial role in nutrient cycling and availability in agricultural ecosystems. While the conversion of agroecosystems from conventional to conservation agriculture practices to restore soil organic matter and soil health has been applied globally, the effect and benefits of such conversions in semi-arid environments remain unclear.

The objective of this thesis was to improve our understanding on the effects of seasonal and spatial variation on the drivers regulating soil organic matter turnover in agroecosystems in semi-arid landscapes. To achieve that, soil chemical and biological parameters in four experimental olive orchards subjected to conventional and conservation management practices were sampled. Soil extracellular enzyme activities serve as a valuable indicator to get insights of soil health, since they have the potential to yield insightful information about the responses of microorganisms to its abiotic environmental and their significance for soil processes. The role of microbial communities in agriculture is vital, being primer decomposers of SOM to meet energy demands and regulate nutrient biogeochemical cycles. Another important indicator of soil health is microbial biomass, being the living component of soil organic matter.

The experimental design of this thesis was divided into field study and lab experiments under controlled conditions. The field study included temperature monitoring at the four olive orchards subjected to different management practices, for three consecutive seasons. Lab experiments included potential soil enzyme activity, microbial biomass carbon and soil organic carbon using soil seasonally sampled from the olive orchards. The potential soil enzyme activities of β-glucosidase, β-xylosidase, phosphatase and β-N-acetyl-glucosaminidase were measured fluorometrically, while soil microbial biomass carbon was determined using the chloroform fumigation-direct extraction

method with sieved field-moist soil. For the determination of soil organic carbon, dry soil samples were analysed using the dry combustion method.

Understanding the temperature sensitivity of SOM decomposition in semi-arid soils, like in the Mediterranean basin, would help predict their response to climate change. The findings of this dissertation reveal positive feedback with agronomic management change from conventional to conservation treatment. The results indicate that conservation practices with added organic matter have higher SOC, benefitting the soil C dynamics and biological activity. While enzyme activity is a sensitive indicator of land management-change, it showed higher sensitivity to seasonal variability.

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Table of Contents

I. List of Abbreviations

Acronym	Definition
OC	Organic Carbon
SOC	Soil Organic Carbon
SOM	Soil Organic Matter
TC	Total Carbon
TOC	Total Organic Carbon
IC	Inorganic Carbon
TIC	Total Inorganic Carbon
EE	Extracellular Enzyme
EEA	Extracellular Enzyme Activity
MUB	4-methylumbelliferone
BG	β-Glucosidase
XYL	β-Xylosidase
PT	Phosphatase
NAG	β-N-acetyl-glucosaminidase
CFDE	Chloroform Fumigation – Direct Extraction
SMB	Soil Microbial Biomass
MBC	Microbial Biomass Carbon
T	Tilled
NT	Non-Tilled
PI	Pruning's Incorporation
LI	Legumes Intercropping
ST	Soil Temperature
AT	Air Temperature
SM	Soil Moisture

II. List of Figures

III. List of Tables

1. Theoretical background

1.1 Soil health and organic carbon

Soil is a highly valuable non-renewable resource and an extremely complex ecosystem. It plays a vital role in dead and decaying organic matter recycling, water filtration, and carbon sequestration, while providing biodiversity habitat (Lal, 2016). Lal (2015) defines soil as "*an organic-C mediated realm in which solid, liquid, gaseous, and biological components interact from nanometre to landscape to generate ecosystem services to all terrestrial life*". Despite its essential role, soil has been rapidly degraded globally due to anthropogenic activities, such as intensive agriculture, adversely affecting human and overall ecosystem health (Yang et al., 2020).

Soil health refers to "*the ability of soil to act as a dynamic living system within the constraints of ecosystem and land use, sustaining animal and plant production, and improving water quality while promoting plant, animal and human well-being*" (Doran & Zeiss, 2000; Tahat et al., 2020). It is based on the concept that soil functions as a key ecological system, essential for providing services that support life by regulating plant health, which in turn influences the health of animals and humans (Toor et al., 2021). Soil quality, on the other hand, encompasses both inherent and dynamic soil properties, representing soil productivity and human-soil interactions due to the inexplicable link with land management (Toor et al., 2021). Due to the high spatial and temporal variability of ecosystem services, an extensive range of physical, chemical and biological soil properties have been selected as soil health indices (Nunes et al., 2021).

Soil organic matter (SOM) refers to the perplexing but stable group of organic polymers, formed through the microbial decomposition of organic residues, such as microbes, dead plants and animals (Li et al., 2023; Abbas et al., 2020). It is considered as primary determinant of soil health, due to its crucial role in essential soil ecosystem functions, comprised by both living and non-living elements (soil microbial biomass, particulate and non-particulate SOM etc.) (Dalal et al., 2011). The contribution of OM to soil health is evident through the retention of plant-available nutrients and the promotion of soil structure formation (Johannes & Kleber, 2015).

1.2 Soil health indices

Given the multifunctional and complex nature of soil systems, a range of indicators have been proposed. Even though soil health indicators fall into three main categories (physical, chemical and biological), they often overlap, as many soil properties result from complex interactions of these factors (Lehmann et al., 2020). Indices used to assess soil health and ecosystem services for effective management can be further divide into two categories based on their response time (short-term or long-term) and sensitivity to land-use and agronomic management change. As Bhaduri et al. (2022) note, soil biochemical indicators can respond rapidly to environmental or agricultural management practices change than conservative soil microbial diversity indices, and thus, utilised as early indicators. Microbial activity, for instance, refers to various physiological functions carried out by microorganisms (Bhaduri et al., 2022). For this purpose, soil enzymes serve as a valuable indicator of the soil's microbial health and physiochemical properties, making them useful for assessing the impact of environmental changes on soil fertility (Sardans et al., 2006). Another sensitive biochemical parameter is microbial biomass, which is directly affected by both biotic and abiotic factors, and thus, rapidly responds to soil degradation, recovery and prevailing environmental conditions (Gatica-Saavedra et al., 2023).

1.3 Agronomic practices

The consequences of inefficient or unsustainable land management are most obvious during the last decades, leading to the depletion of many ecosystem services. The adverse impacts of such management deteriorate both natural ecosystems and human health (Stavi et al., 2016). Risks imposed by soil health degradation include contamination of soil and water resources, decline of SOM, erosion of soil fertile layers, loss of resilience to climate change, and loss of biodiversity. It is especially alarming in arid or semi-arid regions, like the Mediterranean basin, where inputs of organic matter (OM) are lower due to constraints imposed by water scarcity. Even though conventional tillage was the main protagonist as a management practice for olive groves, much attention has been drawn to conservation management practices. The shift from conventional to conservation practices promotes the development and use of sustainable agronomic management (Milgroom et al., 2005).

1.3.1 Conventional agriculture

Conventional agriculture applies tillage by shallow mouldboard plowing or harrowing. The aim of this basic agronomic method is the loosening of upper soil layers, with potentially positive effects in the short run, and overall negative effects in the long run (Stavi I. et al., 2016), including several undesirable implications on soil physical, chemical, and biological properties (Vingozzi N. et al., 2019). The positive effects include the increase of surface roughness and control of weeds, while the adverse include erosion, compaction, and loss of soil fertility. In addition to tillage, other conventional practices include the usage of chemicals to manage weeds or chemical soil additives for nutrient uptake.

1.3.2 Conservation agriculture

Organic and conservation agriculture aims to overcome the adverse impacts of conventional tillage. The basic principle of conservation agriculture is the minimization of soil physical disturbances by mechanical operation that preclude soil inversion, granting retention of crop residues at the soil surface (Vingozzi et al., 2019). No-till and the negation of any tillage, is an integral part of conservation agriculture practices. Promotion of organic carbon sequestration and stabilization due to preservation of soil structure are some results of no-till agronomic practices. Another important component of the conservation tillage is the on-site crop residue management. The retention of crop residues has been documented to potentially decrease soil evapotranspiration loss, improving the overall soil quality (Stavi et al., 2016). In addition, crop residue-covered soil could improve the resilience of dry lands to erratic rainfall patterns from climate change (Mubiru et al., 2017). Intercropping is another conservation practice which promotes biodiversity-ecosystem functions relationship (Gurtright & Tiemann, 2021).

1.4 Soil enzymes

Soil enzymes are defined as protein molecules produced and secreted in the soil matrix by plant roots and soil microorganisms due to metabolism (Zuccarini P. et al., 2023). They facilitate and accelerate various biochemical and nutrient-cycling activities crucial to soil functions, and they are regarded as the primary indicators for detecting initial reactions to alterations in soil management (Scott D. E. et al., 2010).

The predictions of soil responses to climate change could be further aided by understanding the temperature sensitivity of soil organic matter decomposition (Adekanmbi et al., 2023). Based on Adekanmbi et al. (2023), there are two key enzyme-driven steps involved in the decomposition of soil organic matter to generate CO_2, extracellular depolymerization and intracellular metabolism. The first step requires extracellular enzymes of microbial, plant, or animal origin to depolymerize macromolecular constituents of soil organic matter, to produce soluble microbial substrates (Adekanmbi et al., 2023). The second step involves numerous intracellular metabolic processes, where substrates are absorbed and broken down by microbial cells, resulting in the release of CO2 (Adekanmbi et al., 2023).

One of the basic characteristics of soil enzymes is their location: intracellular, cell-bound, and extracellular enzymes. Burns (1982) suggested the categorization of soil enzymes based on their location in soil matrix into ten distinct categories, including:

- Active enzymes function inside the cytoplasm of proliferating microbial, animal, and plant cells.
- Enzymes restricted to the periplasmic space of proliferating Gram-negative bacteria (prior to any leakage, through leakage or modified cell walls).
- Enzymes connected to the external part of the viable cell, with their functional sites reaching out into the surrounding environment.
- Enzymes secreted by viable cells as part of the regular cell growth and division, present in the aqueous phase of the soil.
- Enzymes that bond with humic colloids through processes like adsorption, entrapment, or co-polymerization as humic matter forms.

These categories may represent various stages in the life of enzymes (Burns, 1982). Extracellular enzymes can be either secreted by living cells or released as free enzymes when cells disintegrate (Scott et al., 2010). Such enzymes can be absorbed into organic and mineral components or form complexes with humic substances, or both (Scott et al., 2010; Burns, 1982).

Soil enzymes are substrate-specific, while targeting almost all kinds of macromolecules, including proteins, carbohydrates, amino sugar polymers, organic

phosphates, and lignin (Zuccarini et al., 2023). In addition, they are functionally categorized based on their acquisition target (Meng et al., 2020):

- Polysaccharides are targeted by C acquisition enzymes,
- Chitin, protein, and urea are decomposed by N acquisition enzymes,
- Inorganic P contained in P- organic compounds mineralized by P acquisition enzymes,
- Lignin is decomposed by oxidative enzymes

1.4.1 Extracellular enzymes

Extracellular enzymes (EEs) have a significant role in the ecosystem by facilitating the decomposition of SOM to meet the energy demands and nutritional requirements for microbial growth (Luo et al., 2017). They are synthesized and excreted by microbial decomposers to acquire digestible nutrients for their intake when available energy and nutrients are scarce (Luo et al., 2017). From the standpoint of the organisms that produce EEs, their fundamental role in the soil ecosystem is to acquire resources by making nutrients accessible (Zuccarini et al., 2022). Besides plant roots and living microorganisms, extracellular enzymes can be secreted by dead biota following their lysis (Luo et al., 2017). Following the release of soil EEs, they remain active within the soil solution to interact with their substrates, facilitating the breakdown of different reactions (Zuccarini et al., 2023). A temporary complex is formed through the binding of a substrate and the active site of an enzyme, which is then converted into the final product (Zuccarini et al., 2023).

The EEs are involved in biogeochemical processes, such as the stabilization and destabilization of soil organic matter and nutrient cycling (Bell et al., 2013). Through the production of EEs, soil microorganisms break down and transform polymeric organic matter into smaller, soluble molecules, enabling the assimilation of available nutrients (Bell et al., 2013).

1.4.2 Factors affecting enzyme activity

Understanding the intricate dynamics of soil enzyme activity requires consideration of the various factors influencing it. Such influences may derive from environmental and

soil biochemical parameters. This sector delves into the analysis and impact of such factors on enzymatic activity.

Climatic factors

Precipitation and temperature are key factors with major effect on enzymatic and overall microbial activity (Gomez et al., 2020). In climates with strong seasonal patterns such as the Mediterranean, enzyme activity tends to respond promptly.

The effect of temperature on enzymatic activity may be direct or indirect. The direct impacts are resulting from changes in soil temperature, while indirect effects arise from shifts in SOM content or soil pH (Zuccarini et al., 2023). Temperature inside specific ranges act as a catalyst, accelerating the chemical reactions related to enzymes. Such temperature-driven activity augmentation occurs with adequate level of water availability (Zuccarini et al., 2023), a major limiting factor for microbial growth.

Precipitation rate is positively linked to microbial activity. Previous studies reported that it triggers water pulses in dry soils (known as Birch effect), leading to significant increase of enzyme activities. Water scarcity and low soil moisture tend to reduce enzymatic activity due to the mobility restriction of soil microorganisms, enzymes and nutrients. Hydrolytic enzymes are especially sensitive to water availability due to utilization of water to lyse their specific substrates, especially in arid or semi-arid regions (Zuccarini et al., 2023). Based on the current models, water stress induced by climate change is expected to increase in both frequency and duration. The underlying mechanism of climate change on soil EE activity shifts is difficult to interpret due to the intermix of its direct and indirect effects (Henry, 2013).

Soil pH

Soil pH acts as a regulator of soil enzyme activity by significally influencing the composition of microbial communities. By pH induced alterations of microbial communities, it indirectly affects the types and levels of enzymes in soil. Additionally, pH can affect enzyme activity on two levels: by influencing the ionization of acidic or basic groups within the enzyme’s active centre, and by affecting enzyme kinetics through the alteration of enzyme adsorption on solid surface, solubility and ionisation of substrates (Zuccarini et al., 2023). Various enzymes have pH optima, which is the specific pH at which their catalytic actvity reaches its maximum rate, while also presenting different sensitivities to pH changes.

Nutrient availability

Nutrient availability affects enzyme activity by controlling the substrate levels, enzyme synthesis, microbial activity and enzyme stability. The scarcity of specific nutrient increases the activity of enzymes responsible for the reactions involved at making that nutrient available. Conversely, high concentrations of an enzymatic reactions product, can decrease enzymatic activity through feedback inhibition (Zuccarini et al., 2023).

1.4.3 Soil enzyme activity assay

As Nannipieri (2018) alludes, the interest of soil scientists shifted towards soil enzyme activities, since they yield information on soils' ability to implement biogeochemical reactions (Nannipieri et al., 2018). Enzyme assays are intended to measure the rate of a reaction in the presence of the enzyme – catalyst, providing insights regarding the enzyme concentration in soil (Dick W. A., 2011). Over the years, a wide variety of methodologies have been developed for the measurement of soil enzymatic activities, ranging from classic approaches to the most recent innovative molecular techniques.

Laboratory-based detection of soil enzyme activity has the potential to yield insightful information about the responses of microfauna to its abiotic environment and their significance for soil processes. The most common techniques for the measurement of extracellular enzyme activity (EEA) are colorimetric and fluorometric assays, using p-nitrophenol-linked and 4-methylumbelliferone (MUB) substrates, respectively (Luo et al., 2017; Bell et al., 2013). Bell et al. (2013) state that the fluorometric detection of soil EEA is typically more sensitive metric than colorimetric assays on account of the precise detection of fluorogenic compound separation during substrate degradation, rather than absorbance measurement after the separation of chromogenic component at specific wavelength (Bell et al., 2013). Such approaches measure the "maximum potential" enzymatic activities rather than actual in-situ rates of the catalysed, due to the addition of substrates to soil already containing natural substrates in unknown concentrations reactions (Luo et al., 2017; German et al., 2011; Nannipieri et al., 2018).

1.4.4.1 Fluorometric measurement of extracellular enzyme activity

The usage of fluorometric substrates was first reported by Pancholy and Lynd in 1972, for soil lipase activity calculation (Nannipieri et al., 2018). The development and

utilization of fluorescent dye-conjugated substrates for the quantification of EEA has been a significant advancement in soil enzyme determination, since the 1990's (German et al., 2011). Such developments assist in experimenting with a wider range of hypotheses concerning the role of hydrolytic EEs in soil biogeochemical cycling.

The basic principle of fluorescence enzyme assay involves the addition of synthetic substrates bound to a fluorogenic moiety to soil samples. The bond between fluorescent dye and substrate breaks in the course of substrate degradation, indicating enzyme activity, commonly quantified through the detection of fluorescence dye intensity using a microplate reader or fluorometer (Bell et al., 2013). To ensure the proper estimation of the potential soil EEA, essential parameters must be established prior to the assay conduction.

Assay buffer and pH

Despite the sensitivity of enzymes and their specific pH optima, a majority of the time they do not function at their optimum pH in the ecosystem. The chosen pH used in an assay is determined based on the researchers' questions and hypotheses (German et al., 2011). The pH of such assays is controlled using an aqueous buffer. In literature, there is a wide range of buffers used in the enzyme assays, including sodium acetate, phosphate, and Tris buffers (German et al., 2011; Bell et al., 2013). In some instances, water was suggested as an alternative to buffer. It is worth mentioning that the substitution of buffer by water can potentially cause pH fluctuations.

The buffer-to-sample ratio is another crucial variable, affecting the homogenates' turbidity and the overall fluorescence measurement (German et al., 2011; Bell et al., 2013). Increased concentration of particulate matter will cause "quenching" of fluorescence, while over-dilution may result in undetectable fluorescence.

Soil quenching

As abovementioned, quenching in soil enzyme assays refers to the reduction of fluorescence intensity caused by OM or fragments in the soil slurry during incubation (Bell et al., 2013). It is crucial to run quenching standards concurrently with samples to account for the background fluorescence.

Assay incubation temperature

In addition to the temperature dependence of fluorescence (Nannipieri et al., 2012), soil enzymes present varying temperature sensitivity and optima, depending on

season and latitude (German et al., 2011; Bell et al., 2013). Thus, for site-specific EEA activity, incubation temperature needs to reflect field site values.

NaOH addition

A crucial factor for EEA assays based on MUB-conjugated substrates is the exhibition of optimal pH (>9) for the fluorescence of the released dye to peak. To achieve this pH optima, several protocols suggest the addition of NaOH approximately one minute prior to measurement, to reduce analytical differences. The response of different buffers to the addition of NaOH varies, so different concentrations are needed based on the chosen buffer and its pH (German et al., 2011).

The additional analytical errors that could occur due to the varying substrate fluorescence over time sparked the argument whether the addition of NaOH is necessary (Bell et al., 2013). It is suggested to avoid the addition of NaOH to the enzyme assay if the fluorescence levels are adequately detectable (Bell et al., 2013).

Substrate concentration

Another important parameter of soil enzyme assays is substrate concentration. The fluorometric measurement of potential EEA calls for saturating substrate concentrations to ensure that substrate concentration does not limit the activity (Dove et al., 2020). Due to the especially high variability of soils, assays studying effects of sites or treatments need to be conducted with saturating substrate concentrations to be able to detect any differentiation (German et al., 2011). German et al. (2011) showcased an example of assay using saturated and unsaturated substrate concentrations, presented in Figure 1.

1.4.4.2 Enzyme substrates utilized in herein thesis

Given the importance of substrate specificity in enzymatic reactions, the following section outlines the hydrolytic substrates utilized in the assay and their roles in soil C, N, and P-cycles.

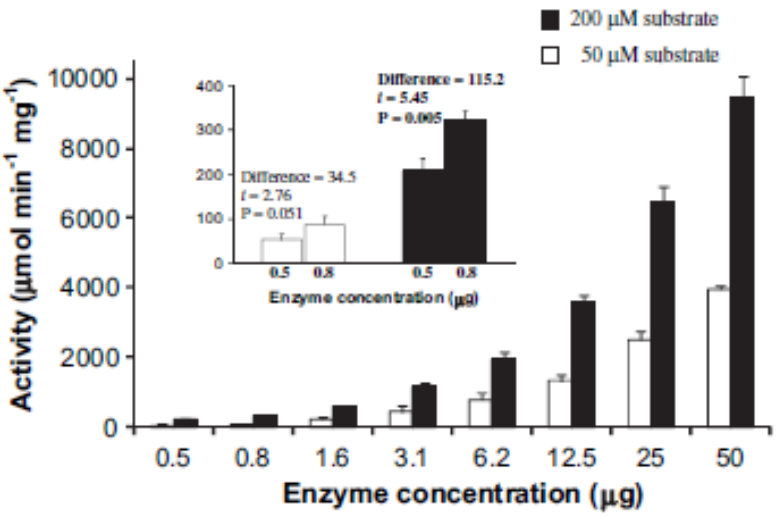

Figure 1. Enzyme concentration of purified β-glucosidase at two substrate concentrations (German et al., 2011)

Table 1: Extracellular enzymes with their corresponding substrate and nutrient cycle.

Enzyme	MUB-linked substrate	Nutrient Cycle
β – Glucosidase	4-MUB-β-D-glucopyranoside	C decomposition
β – Xylosidase	4-MUB-β-D-xylopyranoside	C decomposition
Phosphatase	4-MUB-phosphate	Cleaving of PO_4 from P-containing matter
N-acetyl-glucosaminidase	4-MUB-N-β-D-glucosaminide	Hydrolyzes chitin

β-Glucosidase

β-Glucosidase (BG) is a primary and widely occurring hydrolytic enzyme (Das & Varma, 2011; Bakshi & Varma, 2011). It is one of the most reported soil enzymes in literature while being suggested as an indicator of management effects on soil (Scott et al., 2010). Although no single enzyme activity can fully represent soil metabolic functioning, BG is sensitive to changes in soil and residue management and serves

as an early indicator of changes in soil organic carbon (SOC), often before these changes are detected in physiochemical analyses (Scott. et al., 2010).

It has a crucial role in soils as it catalyses the hydrolysis and biodegradation of various glucosides found in decomposing plant debris within the ecosystem enzyme (Das & Varma, 2011; Bakshi & Varma, 2011). BG is one of the key enzymes for the degradation of cellobiose to glucose (Sinsabaugh et al., 2008; Almeida et al., 2015). Specifically, it facilitates the hydrolysis and biodegradation of β-glucosides, acting in the last rate-limiting stage of cellulose degradation process, the most abundant polysaccharide in soil ecosystem (Adetunji et al., 2017; Scott et al., 2010). The final product of this reaction is glucose, a vital C energy source of soil microorganisms.

β-Xylosidase

β-Xylosidase (XYL) is a crucial extracellular enzymes that facilitate the breakdown of organic C, through hydrolysis of xylooligosaccharides (Xu et al., 2021) and xylobiose. Specifically, XYL is involved in the hemicellulose degradation, a major element of plant cell walls. XYL is present in most xylanolytic systems with the highest reported production deriving from fungi.

Phosphatases

Phosphatases (PT) are a group of abiontic enzymes involved in P-cycle, facilitating the hydrolysis of phosphoric acid esters and in some cases phosphodiesters, and the release of inorganic P necessary for plants and soil microbiota (Sinsabaugh et al., 2008; Adetunji et al., 2017; Stegarescu et al., 2021). The main producers of PT in soil ecosystems are plant roots and microorganisms, such as mycorrhizal and sapotrophic bacteria and fungi (Margalef et al., 2021). Phosphatases are divided into two enzymatic groups: acid and alkaline phosphatases, prevailing in acidic and alkaline soils, respectively (Luo et al., 2017). Observations of alkaline PT are associated only with microorganisms, whereas acid PT was found in most organisms (Luo et al., 2017). In the rhizosphere, acid phosphatases are associated with the decomposition of organic P and the uptake efficiency of P by plants (Ai et al., 2023).

The level of PT in the soil fluctuates based on soil physiochemical properties (e.g. pH, soil temperature, soil water content, soil texture), soil microbiota, the presence of SOM, and agronomic management (Adetunji et al., 2017; Margalef et al., 2021; Stegarescu et al., 2021). Additionally, low soil inorganic phosphorus content and

mineralization of organic P were linked to PT activity in several studies (Janes-Bassett et al., 2022).

β-N-acetyl-glucosaminidase

The hydrolytic extracellular enzyme β-N-acetyl-glucosaminidase (NAG) is regarded as important in both N and C cycling, due to the participation in the conversion of chitin and other β-1,4-linked glucosamine polymers (Sinsabaugh et al., 2008; Uwituze et al., 2022). In many instances, NAG is used as soil chitinase, and N acquisition indicator since it decomposes aminopolysaccharides, specifically chitin and peptidoglycan (Ai et al., 2023; Piotrowska-Dlugosz et al., 2022; Luo et al., 2017).

NAG activity is found to be related to environmental factors such as soil temperature, pH, soil nutrient content, and chemical composition of SOM (Piotrowska-Dlugosz et al., 2022; Luo et al., 2017). Moreover, as Uwituze et al. (2023) mention, NAG is positively correlated to fungal populations, which contain chitin in their cell walls.

1.5 Microbial biomass

Soil microbial biomass (SMB) is defined as the living fraction of SOM (Rice et al., 1996), composed by bacteria, fungi, and other micro fauna communities. It has a key role in nutrient conversion in soil ecosystems by acting as both source and sink of available nutrient (Bhaduri et al., 2022). Similar to soil enzyme activity, SMB serves as a sensitive indicator reflecting on the extent of biogeochemical changes in C stability that occur from soil management change and environmental factors (Moore et al., 2000; Li et al., 2018; Das et al., 2023). Besides the land-use change sensitivity, SMB is sensitive to seasonal variations of soil temperature and moisture, controlling its seasonal dynamics especially in agro-ecosystems (Chernov & Zhelezova, 2020). Due to the involvement of SMB in the decomposition and cycling of SOM (Zhang et al., 2017; Das et al., 2023), the nutrient availability and cycling is predominantly dependent on SMB size and activity (Moore et al., 2000).

1.5.1 Estimation of microbial biomass

The potential influence of SMB can be assessed by its size (Bailey et al., 2002), both directly and indirectly. The direct methods for measurement of SMB involve microscopic procedures (plate counting, biovolume estimation etc.) while indirect once include among others fumigation-incubation and fumigation-extraction methods, and substrate- induced respiration (Moore et al., 2000; Bailey et al., 2002).

Chloroform fumigation-extraction (CFE) method is among the most widely used methodologies for the determination of SMB. The methodology is based on the lysis of soil microbial cells caused by the exposure of samples in chloroform saturated atmosphere. Soil microbial biomass C (MBC) is calculated as the TOC difference of fumigated and non-fumigated soil subsamples, divided by the correction factor.

1.6 Study purpose

The herein study aims to evaluate the coupled effect of lang-management change and seasonality on the drivers regulating soil organic matter cycling in semiarid agroecosystems. The main objective of this study is the investigation of the effects of agronomic practices shift (conventional to conservation) and seasonal variation on soil biochemistry, through sensitive soil indicators. To achieve the abovementioned objectives, soil enzyme activity and microbial biomass C were monitored in selected olive orchards located in West and Central Crete, subjected to conventional and conservation management.

2. Experimental procedure

2.1 Site description and experimental design

For the elaboration of the experimental procedures, surface soil samples were collected from four experimental olive orchards in western and Central Crete. Soils were sampled from 0-15 cm depth from each site during three different seasons: Autumn (October 2022), Winter (February 2023) and Spring (April 2023). The samples were taken from two locations, canopy and rows, for each experimental field under different agronomic practices. The agronomic practices used for the management of experimental sites include both conventional and conservation farming. The conventional practice examines tillage, while the conservation practices include non-tillage, pruning incorporation, and legume intercropping (Table 2). For all sites except Site 3, the conventional treatment is considered as control and includes ploughing once a year.

Figure 2: Map of Crete with indications for the Experimental Sites.

Experimental Site 1

Experimental Site 1 is located in the municipality of Gortyna, in the prefecture of Heraklion in Central Crete (35°05'25.0"N 25°01'01.6"E). It was planted in 2002 with olive trees and has been shifted to conservation tillage since 2021. In the olive orchard under conservation agronomic practice no-tillage is applied, while the weeds are mechanically destroyed and maintained within the field.

Experimental Site 2

Similar to Experimental orchard 1, Experimental Site 2 is located in the municipality of Gortyna, in the prefecture of Heraklion in Central Crete and it was also shifted into conservation tillage in 2021. The utilized agronomic practices are mechanical ploughing (conventional) and no-tillage (conservation).

Experimental Site 3

Experimental Site 3 is located in the Park of Flora Preservation, in the prefecture of Chania in western Crete (35°31'53.3"N 24°03'33.0"E). The olive trees of Site 3 are considered matured, as they were planned in 1970. The field has not been tilled, fertilized nor subjected to grazing in the last 30 years. The orchard has been shifted to organic farming in 2021 with trimming of prunings application to the soil.

Experimental Site 4

Similar to Site 3, Experimental Site 4 is located in the prefecture of Chania in western Crete (35°20'33.7"N 24°17'35.9"E). Besides the conventional tillage, part of the orchard was converted to conservation management 10 years ago. Overall, the field includes three treatments: conventional ploughing, no-tillage combined with incorporation of pruning residues and no-tillage combined with pruning residues incorporation and legumes intercropping.

Table 2: Summary of the agronomic practices used in each of experimental fields.

Field	Treatment	Details
Experimental Site 1	Conventional	Mechanical ploughing
	Conservation	No-tillage
Experimental Site 2	Conventional	Mechanical ploughing
	Conservation	No-tillage
Experimental Site 3	Conservation	No-tillage with pruning's application
Experimental Site 4	Conventional	Mechanical ploughing
	Conservation	No-till with pruning's incorporation No-till with pruning's incorporation and legumes intercropping

Pre-treatment of soil samples

After the collection of the samples at each site, soil was packaged and transported to the laboratory. For the pretreatment of the samples, rocks, leaves, and root material were removed. The soil was then homogenized by gently breaking soil aggregates by hand and passing it through a 2 mm sieve. Half the quantity of freshly sieved samples was stored in the freezer for further biological analysis, where the other half was placed in an incubator at 60^{o}C for drying. The dry soil was then used for physiochemical characterization.

2.2 Physiochemical analysis

2.2.1 Temperature

Temperature was monitored with high resolution at different depths and locations in each of the experimental sites (Table 3). The soil temperature was monitored with 5 minutes intervals.

Table 3: Legend of temperature depth and location monitoring

Location	Depth
Air	-
Below canopy soil surface	0-3 cm
Below canopy	10 cm
Between rows soil surface	0-3 cm
Between rows	10 cm

2.2.2 Gravimetric moisture content

Moist soil samples were weighted right after pre-treatment and then incubated at 60°C for 2 days to dry. Their water content was calculated as:

$$Water\ content\ [\%] = \frac{(weight\ of\ moist\ soil\ [g] - weight\ of\ dry\ soil\ [g]\ )}{weight\ of\ dry\ soil\ [g]} \cdot 100\%$$

2.2.3 Soil organic carbon

Soil organic carbon was measured using SSM-5000A Shimadzu, in the Laboratory of Environmental Engineering and Management. Soil samples were dried at 60°C, homogenized and passed through sieve with 0.25 mm pore diameter. The samples were then analyzed to calculate the difference between total carbon (TC) and inorganic carbon (IC), thereby calculating total organic carbon (TOC).

$$TOC\ [\%] = TC - IC$$

2.3 Biological analysis

2.3.1 Soil enzyme activity

The applied methodology for the measurement of maximum potential soil extracellular enzyme activity was based on the protocol of Bell et al. (2013), including some adaptations. The soil enzyme activities of β-N-acetylhexosaminidase, β-xylosidase, phosphatase, and β-glucosidase were measured for each soil sample, with one repetition. Due to the lack of a plate reader, the experiment was conducted using 1.5 ml tubes (Fig. 3) for the incubation and 0.5 ml tubes for the fluorometric measurement. To avoid timing error, the substrate and MUB were added with specific time delay (10 seconds). These time intervals correspond to the required time by the fluorometer to measure the sample as well as to replace the sample with the next one. This way, each “well” is measured at the exact time, as it would using a microplate reader.

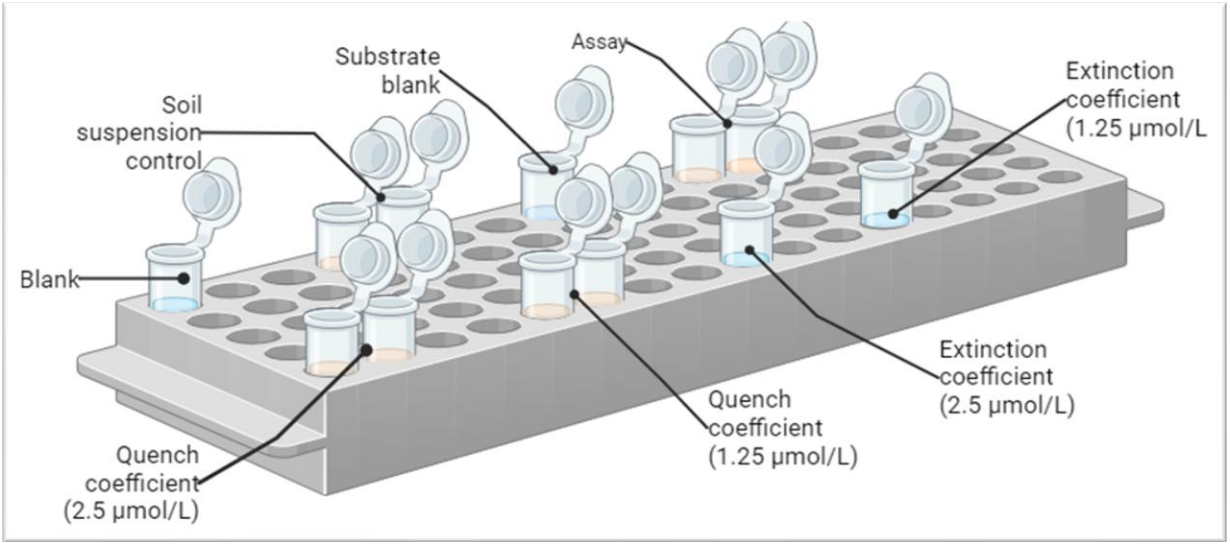

Figure 3: The enzymatic assay set-up representing a microplate.

Preparation of soil suspension

For the slurry preparation, soil samples were weighted (1.2 g) and homogenized in 40 ml of sodium acetate buffer (Na-Acetate, 50 mM) using an ultrasonic bath for 5 minutes, to destroy any soil aggregates. Then, the falcon tubes (50 ml) were placed on a horizontal shaker.

Controls and assay set-up

The chosen high-throughput assay uses MUB, which is a synthetic fluorescent indicator. To account for background noise in the assay reagents and the conversion of fluorescence into correct values, the controls needed to run simultaneously with the enzyme assay (German et al., 2011). In addition, the volume of the controls, blanks, and assays were scaled up in comparison to other protocols. To be specific, the final volume of each Eppendorf tube during incubation was 500 µl (400 µl of soil slurry with 100 µl substrate) compared to the final volume of 250 µl (200 µl soil slurry with 50 µl substrate) used in most papers. The scale up was done to avoid soil particulates during the final pipetting before the recording of fluorescence intensity (Bell et al., 2013). In addition, deviation in volume during the recording of fluorometer will crucially affect the fluorescence (Bell et al., 2013).

The assay set-up was as follows:

- Blank: 500 µl buffer
- Soil suspension control: 400 µl slurry + 100 µl buffer
- Substrate blanks: 400 µl buffer + 100 µl enzyme substrate
- Assay: 400 µl slurry + 100 µl enzyme substrate
- Quench coefficient:
  - 2.5 µmol/L: 400 µl slurry + 100 µl MUB
  - 1.25 µmol/L: 450 µl slurry + 50 µl MUB
- Extinction coefficient:
  - 2.5 µmol/L: 400 µl buffer + 100 µl MUB
  - 1.25 µmol/L: 450 µl buffer + 50 µl MUB

The controls and assays were incubated each time for one hour at 25 ^{o}C. After the incubation, 250 µl of supernatant from each tube was then transferred into a new 0.5 ml PCR tube and measured individually using DeNovix QFX Fluorometer. The excitation/emission was set at 360/465 nm.

Calculation of enzyme activity

The calculation of enzyme activity was conducted based on the paper of German D. P. et al., 2011, “Optimization of hydrolytic and oxidative enzyme methods for ecosystem studies”.

$$Activity\ [nmol \cdot g^{-1} \cdot h^{-1}] = \frac{Net\ Fluorescence\ \cdot Buffer\ Volume\ [ml]}{Emission\ Coefficient\ \cdot Homogenate\ Volume\ [ml] \cdot Incubation\ Time\ [h] \cdot Soil\ Mass\ [g]}$$

Where,

$$Net\ Fluorescence = \left(\frac{Assay - Soil\ suspension\ control}{Quench\ Coefficient}\right) - Substrate\ control$$

Quench and Emission standards were conducted for two concentrations, resulting in the usage of standard curve slopes for the calculation of Quench and Emission coefficients.

$$Quench\ Coefficient = \frac{Slope\ of\ standard\ curve\ in\ presence\ of\ homogenate}{Slope\ of\ standard\ curve\ in\ presence\ of\ buffer}$$

$$Emission\ Coefficient\ [Fluorescence] = \frac{Standard\ curve\ slope\left[\frac{Fluorescence}{\frac{nmol}{ml}}\right]}{Standard\ Volume\ [ml]}$$

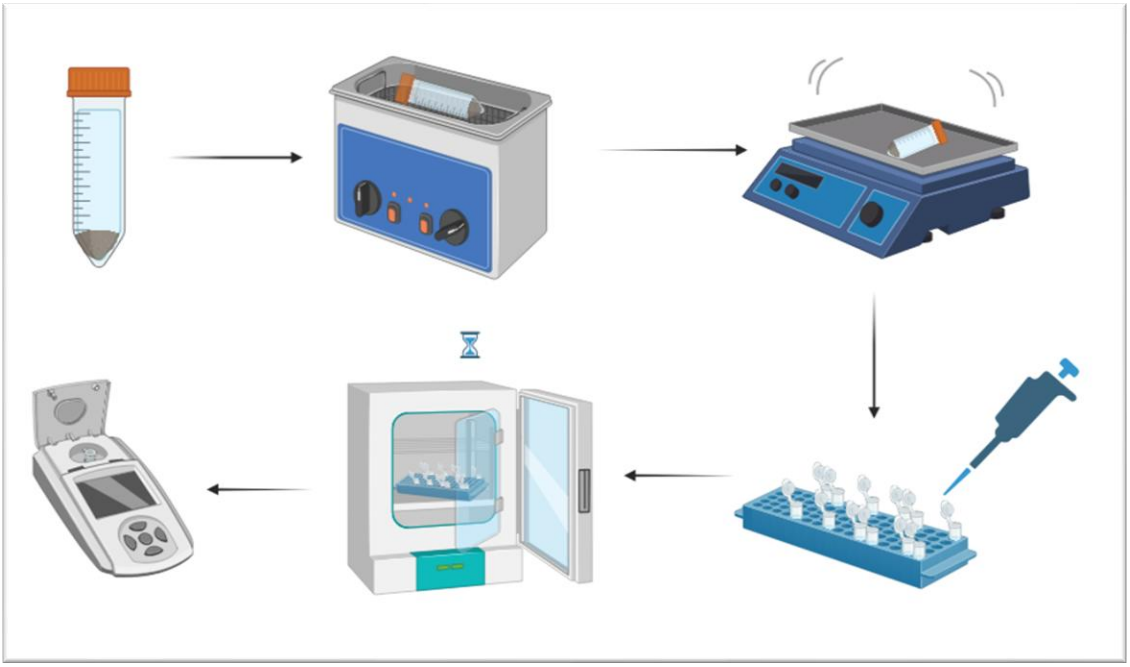

Figure 4: Schematic representation of the enzyme activity methodology.

2.3.2 Microbial Biomass Carbon

Different direct and indirect methodologies have been proposed to measure microbial biomass carbon. The chosen methodology for the elaboration of the dissertation was Chloroform Fumigation – Direct Extraction (CFDE), based on the “Allison Lab protocol: Microbial Biomass by fumigation” (Allison S., 2008), including some variations.

The CFDE methodology is divided into two parts: the fumigation of samples using chloroform and their extraction using K2SO4, and the direct extraction of non-fumigated samples using K_2SO_4 (control). For this reason, each soil sample is divided into two subsamples (5 g soil with field moisture) and placed into a 50 ml glass beaker and in a 50 ml falcon tube, respectively. The experimental design for the fumigation part of CFDE is shown in the Figure 5.

Beakers containing soil subsamples were placed into the vacuum desiccator with an additional beaker containing 25 ml ethanol-free chloroform and a dozen glass beads. The desiccator was evacuated for approximately 10 to 15 seconds using vacuum pump or until the chloroform has reached its boiling point. The vacuum valve was left closed for 2 minutes for the chloroform to boil intensely. After the necessary time passes, the valve was opened to let air pass into the desiccator for the uniform distribution of chloroform into the soil. The same evacuation - venting process was repeated two more times, not venting the last time. Both valves on the desiccator and pump remained closed while the samples were left covered with cloth in the dark for three days for the fumigation (Rice et. al, 1996). During the evacuation of the desiccator, grease was used to seal any part that could cause vacuum losses. After three days, the vacuum was released and the beaker containing the chloroform was removed. Vacuum was once again drawn and released to remove excess chloroform.

For the extraction part of the methodology, every subsample was extracted using 20 ml 0.05 M K_2SO_4 (1:4 ratio) and then was shaken for 1 hour. Whatman No. 1 filters were folded, placed in a funnel, and leached with K_2SO_4 prior to the extraction of the samples. Furthermore, extracts were stored in a freezer until their analysis while non-fumigated samples (controls) were extracted immediately and stored the same way. During the storage of subsamples in the freezer a white precipitate occurs, so prior to the analysis, every sample was filtered again using Whatman UNIFLO PVDF

membrane (0.45 μm). The analysis of the samples was conducted using TOC – L analyser (Total Organic Carbon Analyzer, Shimadzy).

The calculation of MBC was based on the equation:

$$MBC = {}^{EC}/_{k_{EC}}$$

where, EC is the difference between C in the non-fumigated and fumigated samples (TOC of fumigated – TOC of non fumigated) and kEC=0.45 (Joergensen, 1995).

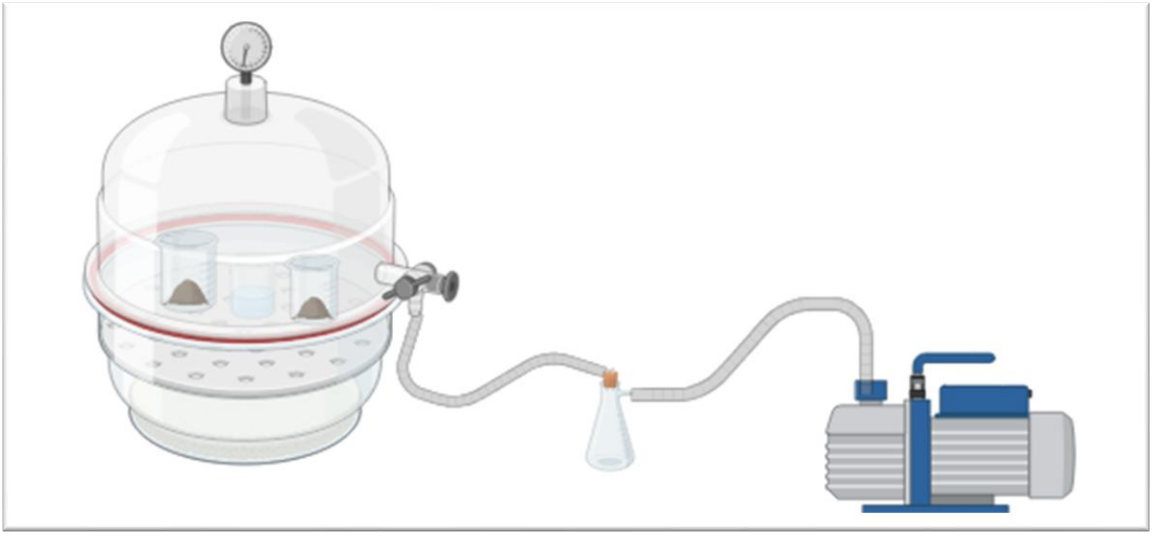

Figure 5: Experimental set-up of the fumigation process for the microbial biomass C assessment.

2.4 Statistical analysis

The statistical analysis was used to assess the influence of each factor (Site, Season, Treatment and Location) and their interactions on soil biological parameters. To achieve that, one- and multi-way analysis of variance (ANOVA) were implemented at a confidence level of 95%. Additionally, Tukey-HSD test was performed for post-hoc comparison between the levels within each considered factor. Correlations were performed using the Pearson method. All statistical analyses were performed in the R Language.

3. Results

3.1 Temperature

Soil temperature (ST) is responsible for the heat energy and mass exchange regulation between soil and atmosphere, influencing the biochemical reaction rates, evapotranspiration, and aeration in soil (Baldrian et al., 2013; Munoz-Romero et al., 2015; Costa et al., 2023). While ST is firmly coupled with air temperature (AT) near surface, they vary due to factors such as solar radiation, precipitation, vegetation, soil depth and soil properties (Zhang et al., 2021).

Soil surface temperature, defined as the temperature in the first 10 cm of soil (Xu et al., 2020), is among the key factors affecting biological processes such as soil respiration, microbial diversity, enzymatic activity, and overall SOC dynamics (Baldrian et al., 2013; Costa et al., 2023). Based on the monitored ST between experimental Site 1 and Site 4, as shown in Fig. 6, it can be observed that even though ST was overall lower for the specific time at Site 1, the variation between the two depths (0-3 cm and 10 cm) was higher in Site 4. The average daily ST for Site 1 was 19.28 ^{o}C and 19.02 ^{o}C, while for Site 4 20.85 ^{o}C and 20.47 ^{o}C, at the respective depths for three months (April 25th – June 21st). The observed variation is attributed to the different soil characteristics (soil texture, structure, organic C content) and climatic conditions in the respective areas (West and Central Crete).

The progressive phase shift of near subsurface temperature has been documented, highlighting slightly higher ST during times with minimum AT, and lower ST during high AT (Nwanko & Ogagarue, 2012). Nwanko and Ogagarue (2012) base this shift of ST on the coupled soil heat transfer, radiation, and surface latent heat exchanges. Such shifts can be observed, for example, in Fig. 7 that displays the daily average, maximum, and minimum temperatures in Site 4 at different depths (AT and 10 cm depth) below an olive tree canopy. During the colder months of winter, higher average daily ST were documented at 10 cm depth, compared to the monitored ST at 0-3 cm.

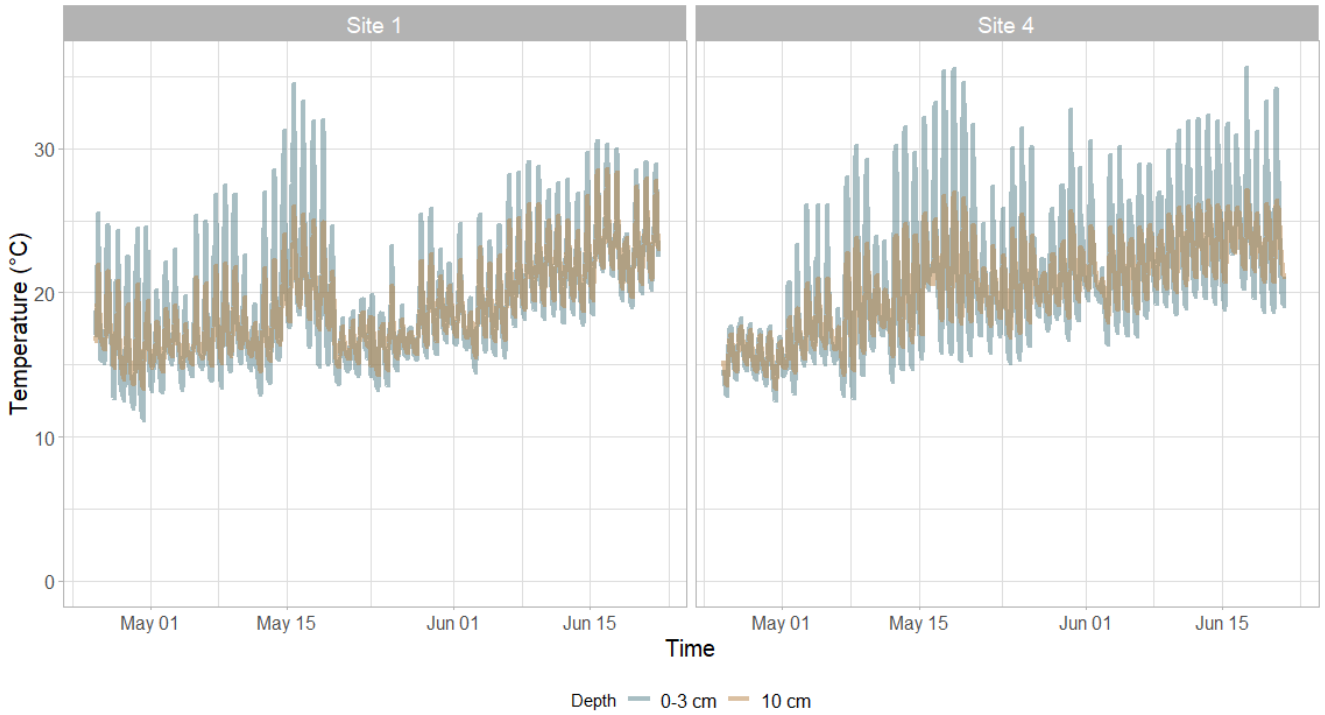

Figure 6: Monitored soil temperature for Experimental Site 1 (left) and Site 2 (right) at 0-3 cm (grey) and 10 cm (brown) depth. Temperature was documented in 5 minutes intervals.

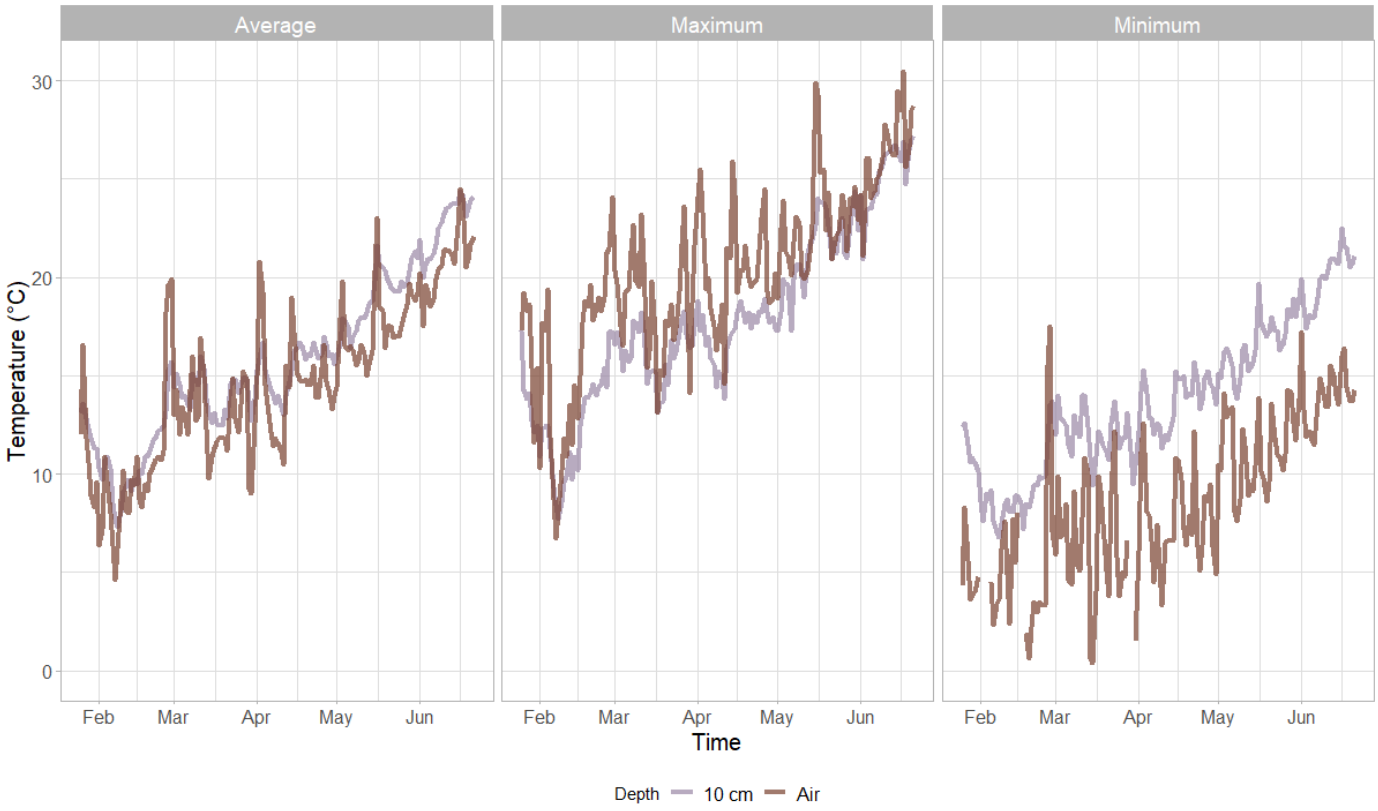

Figure 7: Average (left column), maximum (middle column), and minimum (right column) daily temperature variation at air (brown) and 10 cm soil depth (lilac) in Experimental Site 4.

Beside the differentiation of surface ST between two sites, temperature fluctuations are distinguished regionally. For example, ST below the olive canopy is lower than the temperature measured in the same field but with bare soil and direct sunlight (Fig. 8). Moreover, daily temperature variation was detected in the same experimental field. As Figure 9 highlights, the range of temperature differentiation between the two locations and selected depths vary, even though both sensors were below olive tree canopy.

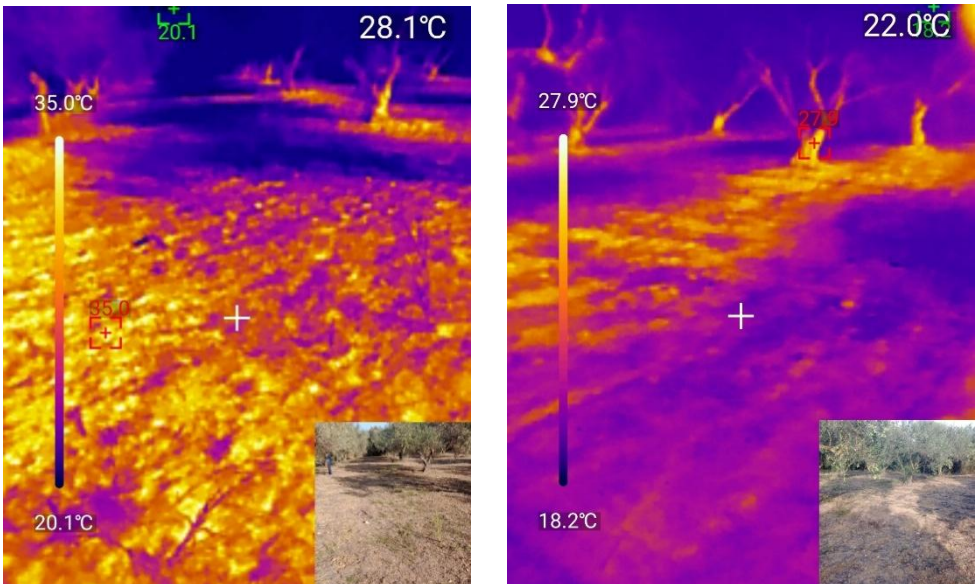

Figure 8: Infrared thermography images of Site 4 highlighting the difference of temperature between air, ST in direct sunlight and ST in the shadows. The data were taken on November 10th 2024 in the afternoon (Credits: Ioannis Kasapakis).

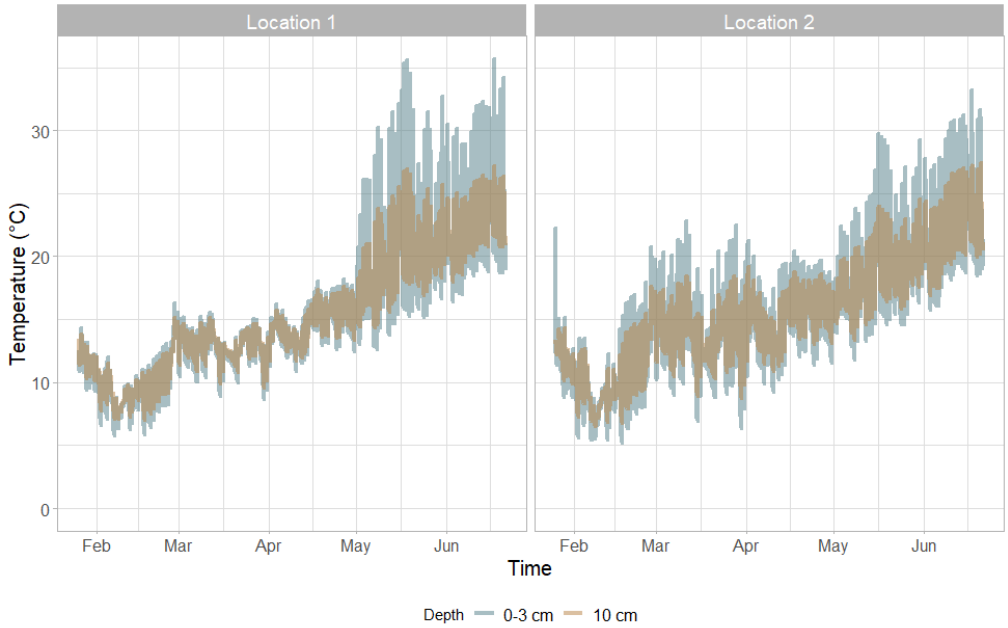

Figure 9: Temperature variation of two locations on the same experimental field (Site 4). The line colour indicates monitored soil temperature at different depths: 0-3cm (grey) and 10 cm (brown). Temperature was logged at 5 minutes intervals.

Soil temperature monitoring is of great significance in the context of SOC dynamics under climate change, being among the drivers of CO_2 emissions due to the linear relationship between soil respiration and ST (Xu et al., 2020). However, said relationship remains somewhat contradictory. Higher soil microbial respiration is anticipated with ST increase which would result in higher CO_2 emissions and further higher temperatures. By contrast, with elevated temperatures C sequestration is promoted through faster microbial OM mineralization, nutrient release, and enhanced plant growth resulting from intense metabolism activity of OM decomposers (Costa et al., 2023).

3.2 Moisture content

Soil moisture (SM) plays a critical role in many ecological processes, influencing biological activities, nutrient cycling, soil structure, hydrology and climatic conditions, especially in regions like the Mediterranean region where SM is considered a limiting factor (Escorihuela & Quintana-Seguí, 2016). Conservation management practices, such as reduced or no-tillage coupled with residue retention on soil surface, could potentially enhance moisture infiltration through the formation of barrier to evaporation, improving the stored moisture stored to root zone (Govaerts et al., 2007).

Gravimetric moisture content was monitored across sites and management practices during winter and spring sampling of 2023. When interactions among the examined factors were not considered, moisture content was primarily influenced by study region (Fig. 10, $p<0.001$). Specifically, moisture content was highest and statistically significant in experimental Site 2 (34.9%) when compared to Site 1, Site 3 and Site 4 (18.4%, 16.5% and 21.7%, respectively). In terms of seasonality, no statistically significant difference was observed between winter (21.1%) and spring (25.3%) ($p=0.274$). Similarly, moisture content did not significantly differ among agronomic practices, with highest content under no-tillage (28.4%) and lowest under no-tillage with pruning's incorporation (18.4%) ($p=0.225$). Likewise, location had no significant effect on moisture content ($p=0.61$) when interactions were not considered.

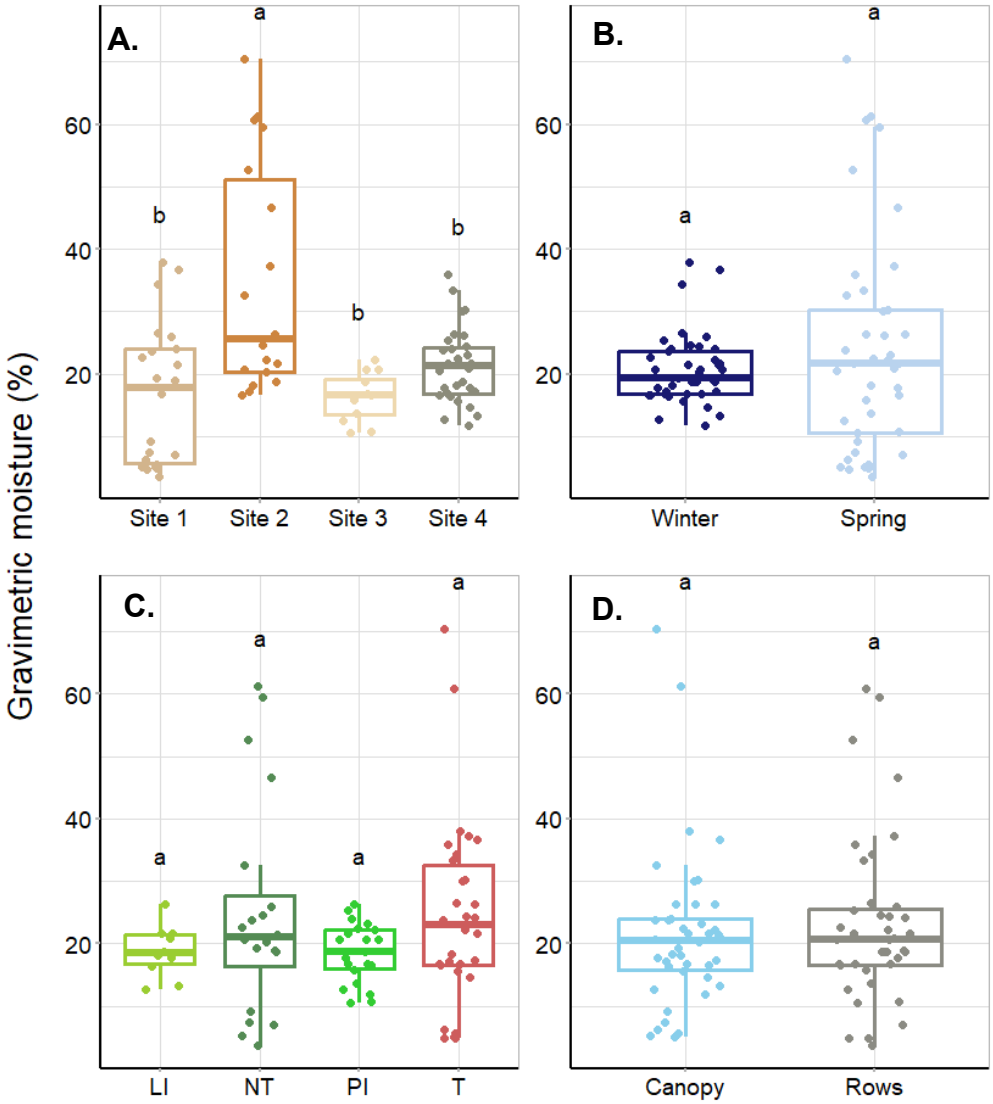

Figure 10: Average moisture content according to the examined factors: A. study region, B. seasonality, C. treatment and D. location. Error bars represent standard error. Different lowercase letters indicate statistical significance based on Tukey's post hoc test ($p<0.05$).

The seasonality of moisture content at the individual study regions under different agronomic management practices can be observed in Figure 11. Besides spring sampling of Site 2, moisture content was below 40% independent of study region, season and agronomic practices. In Site 1, moisture content is higher under conventional than conservation treatment (29% and 22%, respectively) during winter and scarce during spring (5% and 6%) with statistically significant differences. Similar trend can be observed in Site 3 with moisture reaching 20% during winter and 18% in spring, with no statistically significant difference. Contrary to Site 1 and Site 3, Site 2 and Site 4 follow the same pattern, with lowest moisture during winter sampling and higher during spring. Additionally, in Site 2, the average moisture content under no-

tillage is higher and statistically significant different than the conventional management practice independent of season. In Site 4, SM is slightly higher under conservation management practices during winter, while in the case of spring, SM content is higher under conventional treatment.

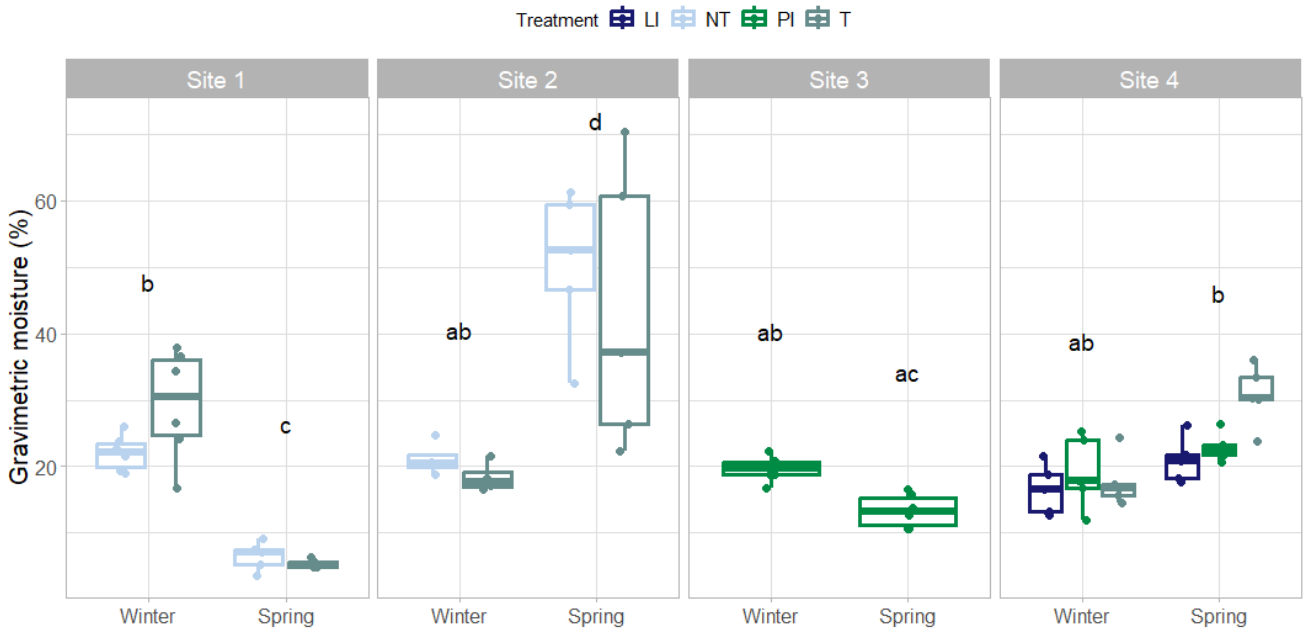

Figure 11: Average moisture content across experimental fields under different agronomic management practices for two consecutive seasons. Error bars represent standard error. Different lowercase letters indicate statistical significance based on Tukey's post hoc test ($p<0.05$).

3.3 Soil carbon stocks

3.3.1 Soil organic carbon

Due to the complex nature of Mediterranean soil and soil OC, results were analysed both in terms of the individual factors influencing SOC and their combined effect. The findings of the present study demonstrate that study region, seasonality and implemented management practice primarily influenced SOC when examined separately. The analysis of SOC content reveals that the average SOC in Site 4 is significantly higher than in the other experimental orchards, with 26.23 g C/kg soil while SOC content for the remaining sites varied between 14.81 g C/kg and 15.62 g C/kg soil (Fig. 12). Furthermore, when SOC content was analysed to investigate the effect of management practices, it indicates that conservation treatments with pruning's incorporation and legumes intercropping positively affected SOC content.

Specifically, although the content of conservation treatments LI and PI were higher, when compared to conventional treatment (control), only LI was statistically significant.

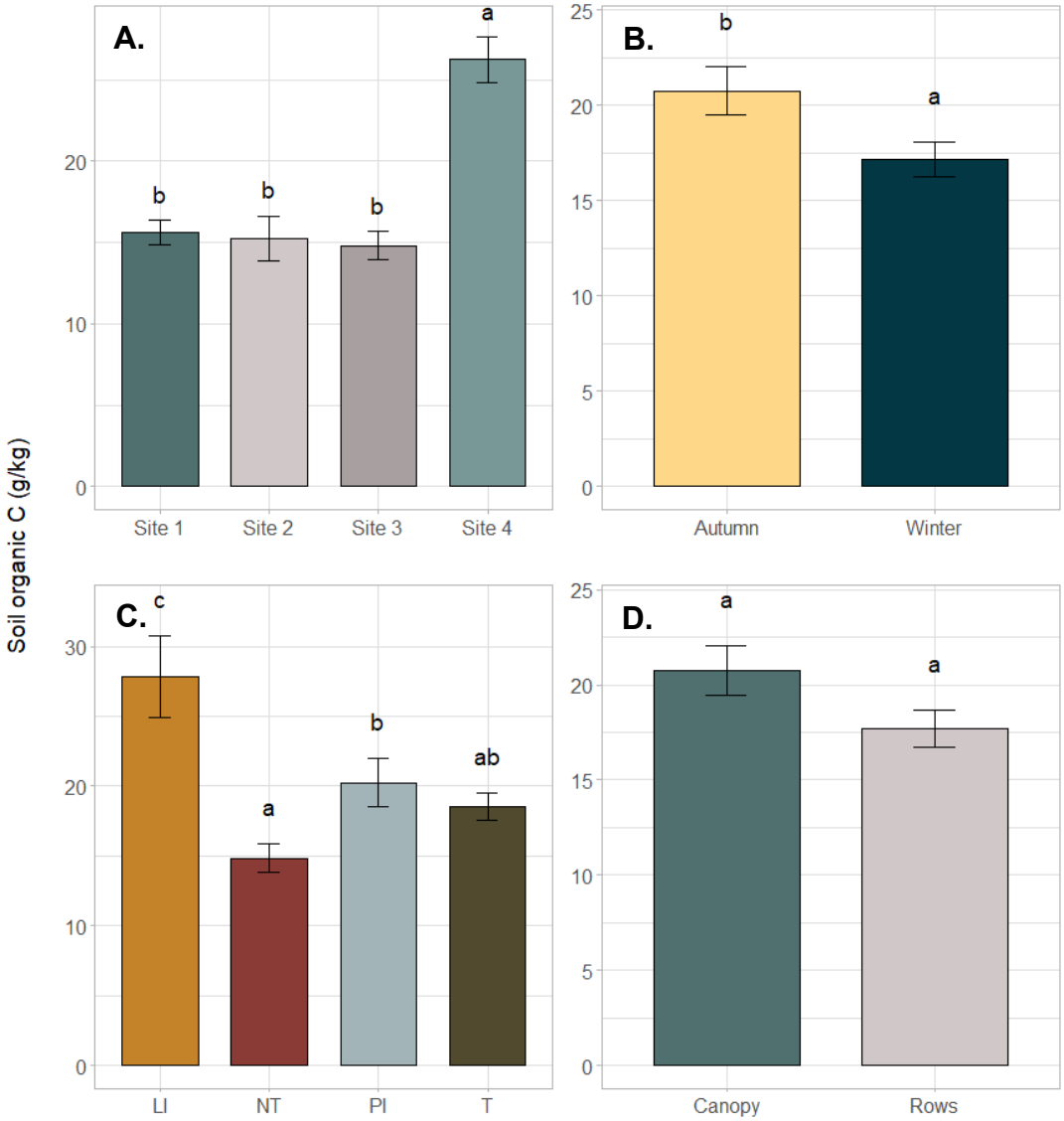

Figure 12: Soil organic c content based on four factors: A. study region, B. season, C. treatment, and D. location without considering any interactions between them. Error bars represent the standard error. Different lower-case letters on top of bars indicate significant difference between the levels of said factor, based on Tukey HSD test ($p<0.001$).

Seasonality also influenced SOC content, with higher average levels during autumn ($p=0.03$, Table 4). Even though SOC content was slightly elevated below the olive tree canopy when compared to inter-rows, the differences were not statistically significant ($p>0.06$).

Table 4: One-way ANOVA results of SOC for each of the studied factors.

Factor	F-value	p-value
Site	23.31	<0.001 ***
Season	4.85	0.03 *
Treatment	8.73	<0.001 ***
Location	3.53	0.064 .

Significance codes: " . " <0.1, " * " <0.05, " ** " <0.01, " *** " <0.001

Table 5: Pairwise comparison results for soil organic carbon, using Tukey HSD test (p<0.05).

Factor	Diff	p adj.
	Site	
Site 2 – Site 1	-0.37	0.997
Site 3 – Site 1	-0.81	0.976
Site 4 – Site 1	10.61	0
Site 3 – Site 2	-0.44	0.997
Site 4 – Site 2	10.98	<0.001
Site 4 – Site 3	11.42	<0.001
	Season	
Winter - Autumn	-3.58	0.030
	Treatment	
NT – LI	-12.99	<0.001
PI – LI	-7.61	0.019
T – LI	-9.28	0.002
PI – NT	5.39	0.042
T – NT	3.72	0.219
T – PI	-1.67	0.809
	Location	
Rows - Canopy	-3.05	0.063

NT = no-till; LI = no-till with pruning's incorporation and legumes intercropping; PI = no-till with pruning's incorporation; T = mechanical plough.

To further evaluate the factors influencing SOC variation, their coupled effect was analysed (Table 6). When the combined effect of seasonality and study region was examined, the statistically significant difference of SOC content during autumn sampling in Site 4 can be observed, whilst the general seasonal variation in Sites 2 and 3 is minor (Fig. 13). As for the seasonal variation of SOC content in orchards under different agronomic management practices, the same trend can be observed for all treatments, with SOC content decrease from autumn to winter (p<0.001, Fig. 14). Pertaining site-specific conservation treatments, the results of experimental sites 1

and 4 indicate SOC enhancement, with average increases up to 3.78 g C/kg in Site 1 under no-tillage, 4.68 g C/ kg under LI and 4.47 g C/kg under PI in Site 4 (Fig. 15).

Excluding the conservation treatments of experimental sites 2 and 3, where slightly higher but not statistically significant SOC contents were observed, the same seasonal pattern was detected regardless of the applied management practice, as it was expected due to the slow respond of SOC (Fig. 16). The highest SOC content was recorded in Site 4 under conservation practices consistently across season, reaching over 30 g C/kg soil for both LI and PI treatments (33.53 g C/kg and 30.74 g C/kg, respectively), compared to 24.59 g C/kg under conventional treatment at the same experimental site. This suggest that the increase in SOC could be potentially attributed to organic input, following the transition from conventional to conservation management. Although a similar pattern is evident in Site 1, SOC content is considerably lower for both conservation and conventional treatments (19.22 g C/kg and 15.51 g C/kg, respectively), reflecting the potential influence of the organic matter input in the form of prunin's incorporation in soil, coupled with climatic conditions and inherited soil characteristics. Contrasting Site 1 and Site 4, SOC content in Site 2 is higher under conventional treatment (20.21 g C/kg soil), whilst also contradicting the seasonal pattern with higher content during winter sampling under NT treatment (14.40 g C/kg soil).

Table 6: Soil organic C multi-way ANOVA results for the interactions of the studied factors.

Interactions	F-value	p-value
Site – Season	14.26	<0.001 ***
Site - Treatment	14.13	<0.001 ***
Season - Treatment	5.83	<0.001 ***
Site – Season – Treatment	9.85	<0.001 ***
Site – Season – Treatment - Location	5.76	<0.001 ***

Significance codes: " . " <0.1, " * " <0.05, " ** " <0.01, " *** " <0.001

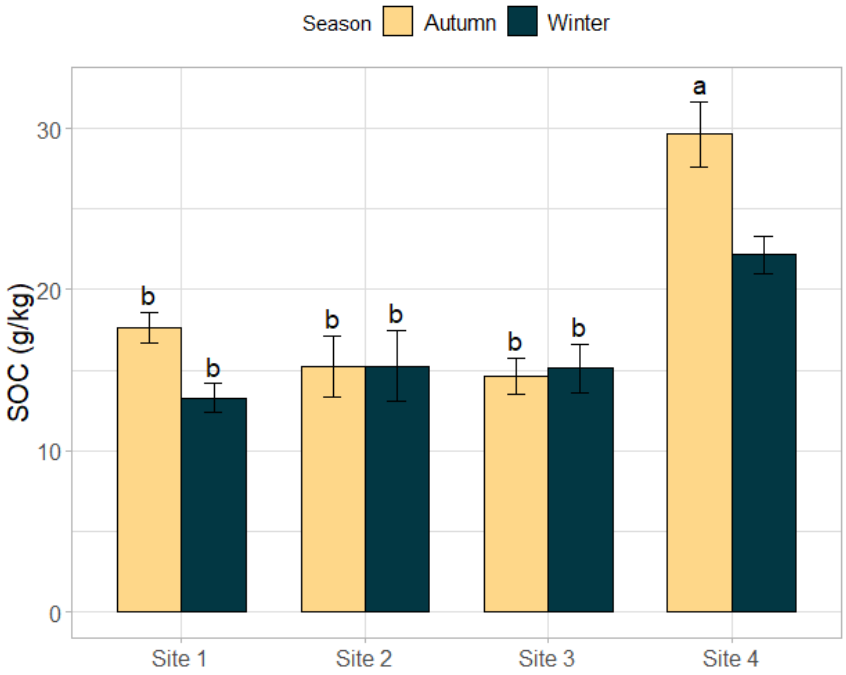

Figure 13: Seasonal variation of soil organic C content under different management practices. Error bars represent standard error. Different lower-case letters indicate statistically significant differences based on Tukey HSD test ($p<0.001$).

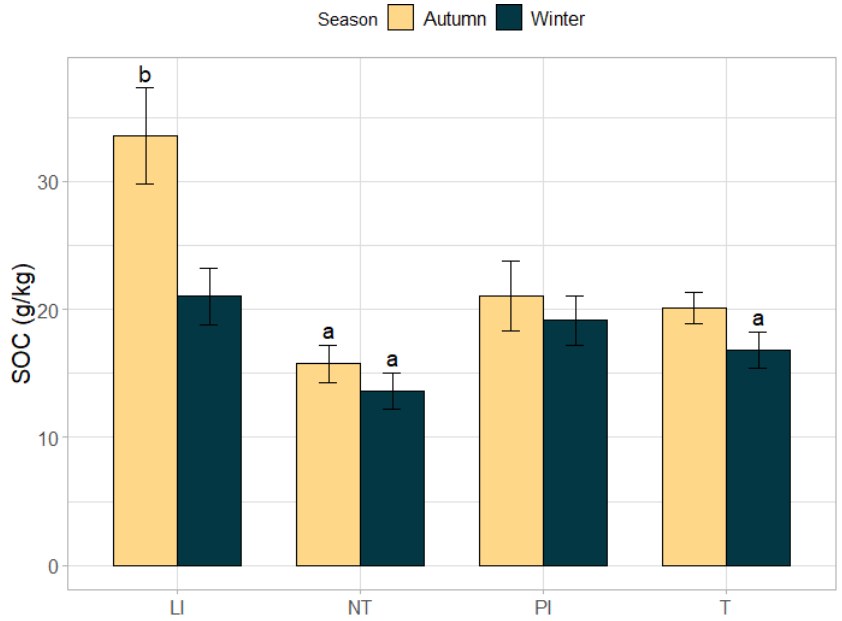

Figure 14: Seasonal variation of SOC stocks across experimental sites. Error bars represent standard error. Different lower-case letters indicate statistically significant differences, based on Tukey HSD test ($p<0.001$).

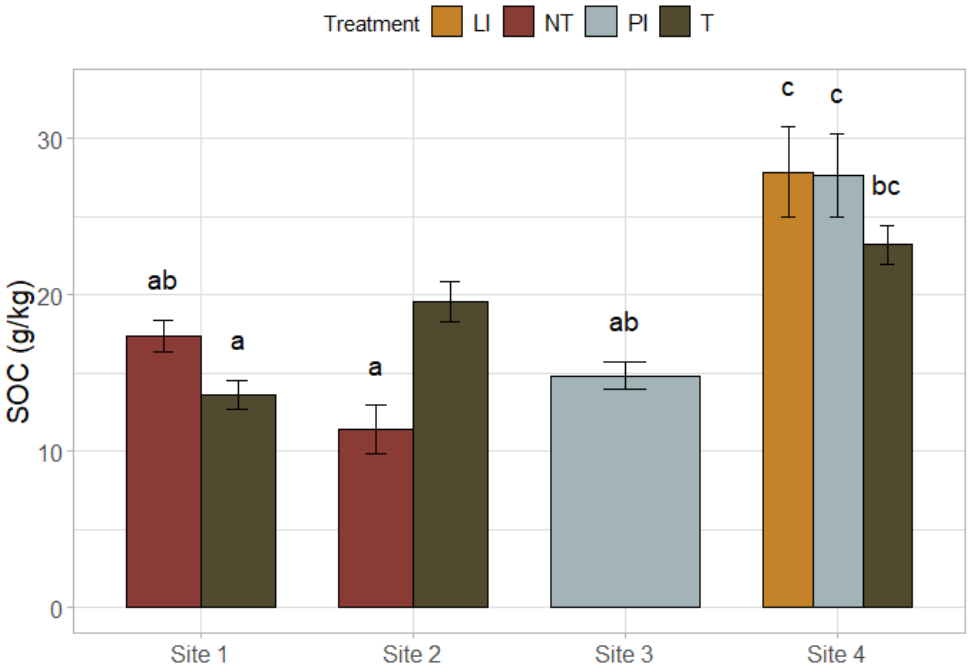

Figure 15: Soil organic C content variation in experimental sites under different management practices. Error bars represent standard error. Different lower-case letters indicate statistically significant differences based on Tukey HSD test ($p<0.01$).

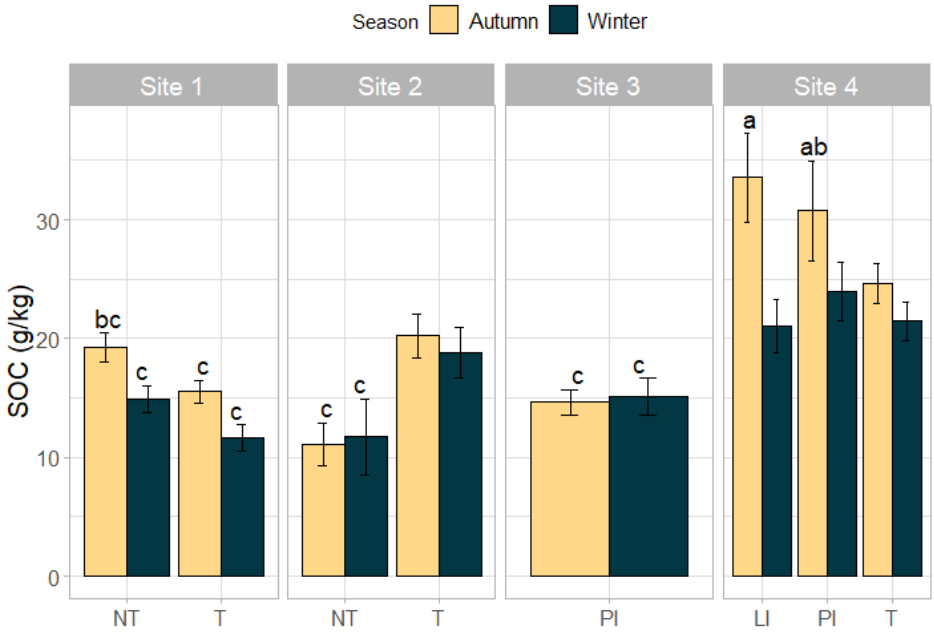

Figure 16: Soil organic C content seasonal variation in experimental sites under different management practices. Error bars represent standard error. Different lower-case letters indicate statistically significant differences based on Tukey HSD test ($p<0.001$).

3.3.2 Soil inorganic carbon

While the response of SOC to land management has been well-documented both regionally and globally, the soil inorganic carbon (SIC) response to such changes remains understudied (An et al., 2019). In Mediterranean basin, a significant portion of agroecosystems is established on soils with low OC levels and varying levels of IC, consisting of different types and concentrations of carbonates (e.g. limestone) (Apesteguia et al., 2018; Leogrande et al., 2021). Although SIC is considered very stable, recent studies highlight the potentially rapid changes through secondary carbonates produced by soil organisms, with higher accumulation rates in arid and semi-arid regions (Raheb et al., 2017).

In the herein study, the analysis of SIC stocks revealed statistically significant differences and site-specific influence ($p<0.001$, F value = 40.53) (Fig. 17, A.). The highest and lowest average SIC stocks were observed in Site 2 (4.52%) and Site 3 (0%) respectively, while the average SIC content in Site 1 and Site 4 were 2.50% and 2.73% accordingly. Besides the study region, SIC variation was observed when analysed based on management practice, where statistically significant and highest SIC was documented under no-till management (Fig. 17, C.). Regarding the coupled effect of study region and management practice, excluding Site 3, SIC was overall higher under conservation treatment, however, in Site 1 SIC was higher under conventional treatment (Fig. 18). SIC was generally higher than SOC across experimental sites, besides Site 3 where no SIC was detected (Fig. 19). Specifically, in experimental Site 1 SIC was 9.40 g C/kg soil higher than SOC, whilst greatest difference was documented in Site 2 where SIC was higher almost 30 g C/kg. In the case of west Crete, no clear pattern can be observed. In experimental Site 4, the difference between SIC and SOC are not that significant, with SIC being only 1.10 g C/kg higher while in Site 3, no IC was detected, probably due to geological factors such as parent material and underlying geology.

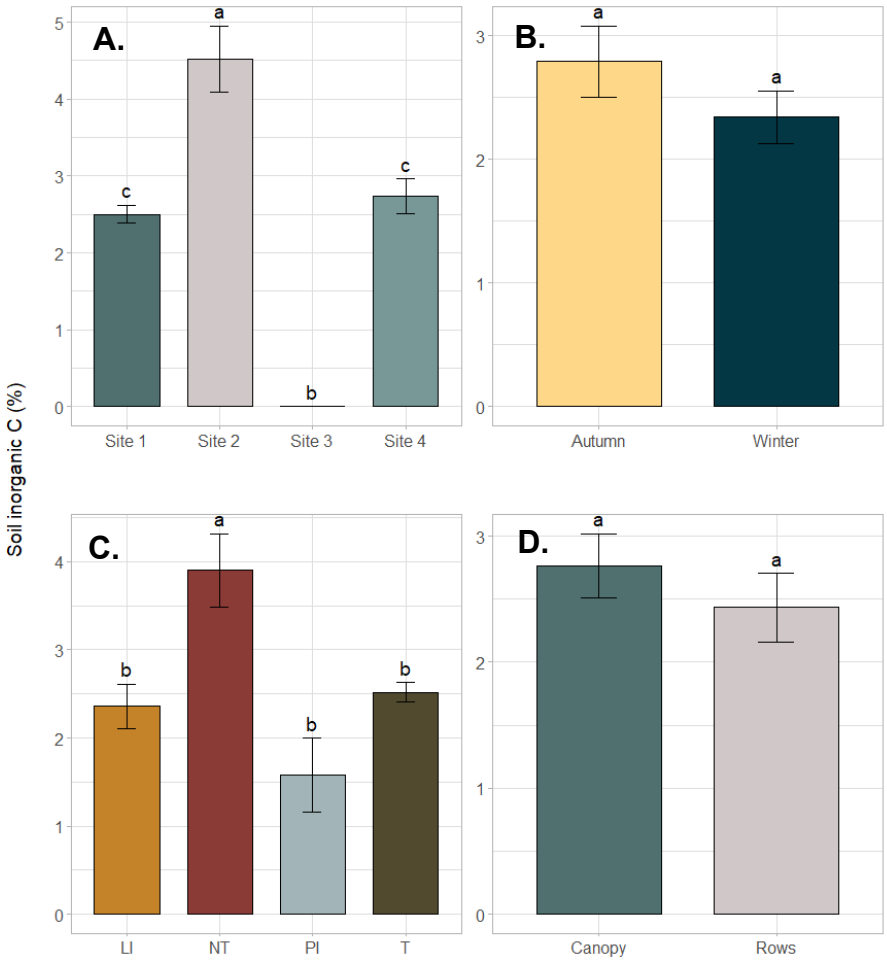

Figure 18: Soil organic carbon content based on four factors: A. study region, B. season, C. treatment, and D. location—without considering any interactions between them. Error bars represent the standard error for each factor at the respective plot. Different lower-case letters on top of bars indicate significant difference between the levels of said factor, based on Tukey HSD test.

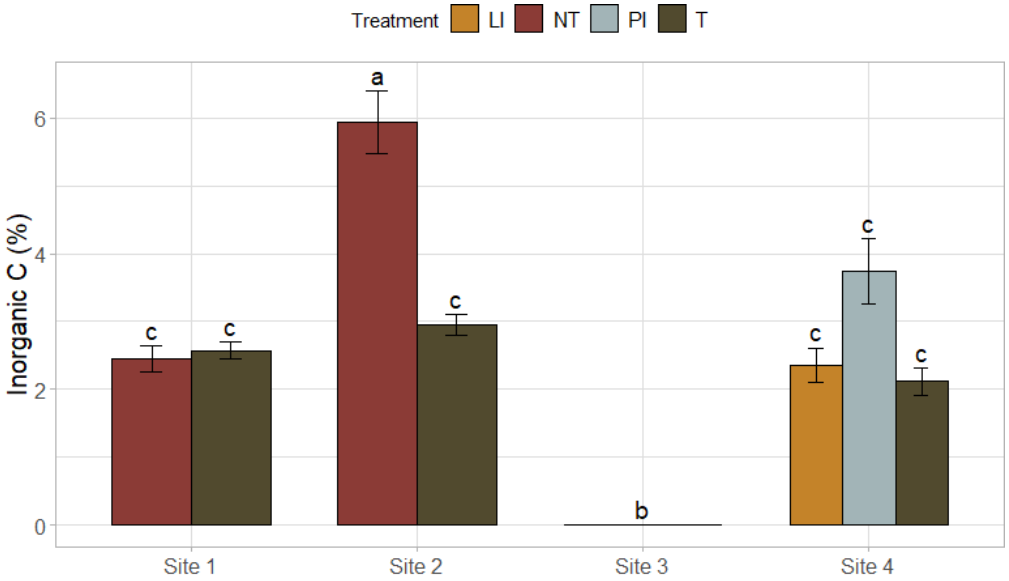

Figure 17: Site variation of soil inorganic C under different management practices. Error bars represent standard error. Different lower-case letters indicate statistically significant differences based on Tukey HSD test ($p<0.001$).

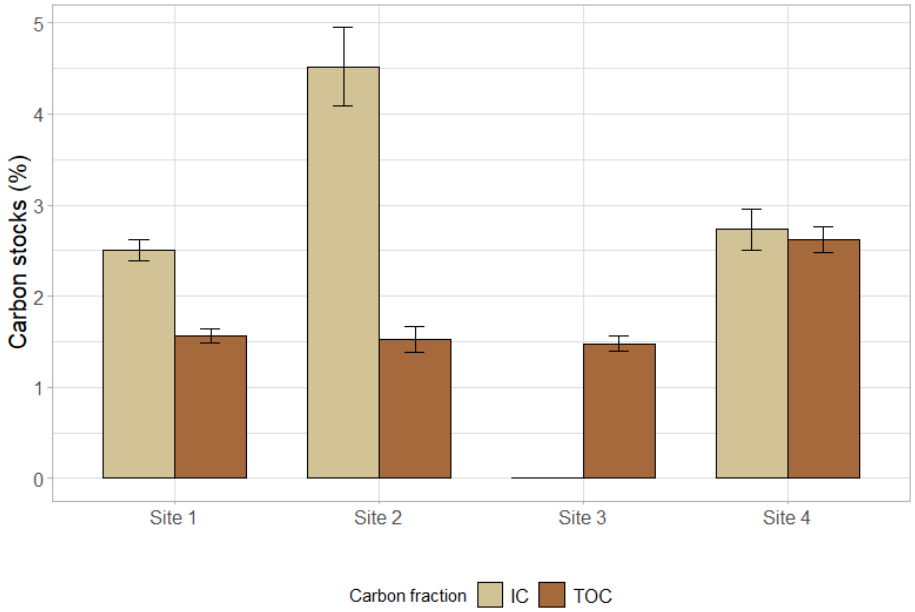

Figure 19: Soil organic and inorganic carbon fractions across experimental sites. Error bars represent standard error.

3.4 Microbial biomass

Microbial biomass is an indispensable driver of soil fertility and nutrient cycling, reflecting the active microbial community in the soil responsible for OM decomposition and nutrient cycling. The magnitude and activity of soil microbial biomass C, which represents the proportion of SOC held withing the living microorganisms, is sensitive to agronomic practices. The following section presents the analysis of MBC in regard to seasonality and agroecosystem management practices, highlighting the effects of these factors on soil biological activity.

Without considering interactions among the examined factors, the analysis of MBC revealed that it is primarily influenced by site, seasonality and management practices. The average MBC in experimental Site 4 (860 mg C/kg) was significantly higher than in Sites 1, 2 and 3 (545.47 mg C/kg, 593.31 mg C/kg and 507.53 mg C/kg, respectively) (Fig. 20). Seasonality favoured microbial biomass during winter (725.85 mg C/kg) and spring (741.63 mg C/kg), with statistically significant differences compared to autumn (550.28 mg C/kg) (Tables 7 & 8). Seasonal changes, such as soil temperature and precipitation, affect MBC through their influence on microbial growth and activity (Das et al., 2023). Das et al. (2023) highlight the seasonal fluctuations and the increase in MBC during wet season. When considering only the effect of management practices on MBC, the conservation treatment LI (no-till with pruning's

incorporation and legumes intercropping) was statistically significant to NT and T. The results based on the management practices agree with earlier studies conducted in the Mediterranean basin, where biological indicators were monitored to evaluate the short-term effects of agronomic practices on biological quality indicators (García-Orenes et al., 2010). Although the average MBC was reported slightly higher below the tree canopy compared to the inter-row samples, the difference was not statistically significant.

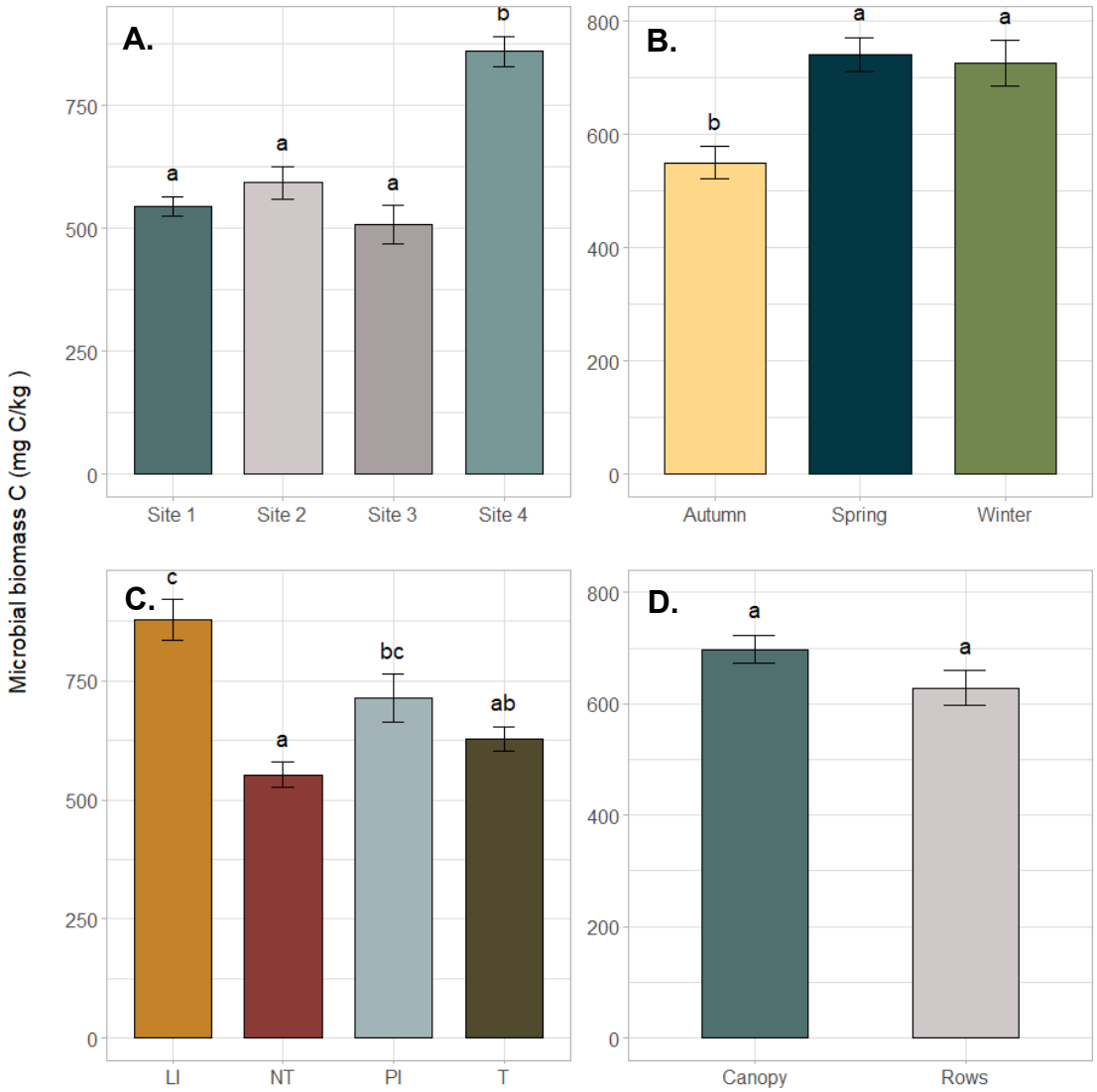

Figure 20: Average microbial biomass C according to the examined factors: A. study region, B. seasonality, C. treatment and D. location. Error bars represent standard error. Different lowercase letters indicate statistical significance based on Tukey's post hoc test ($p<0.05$).

Table 7: One-way ANOVA results for microbial biomass C for each of the studied factors.

Factor	F-value	p-value
Site	30.52	<0.001 ***
Season	11.36	<0.001 ***
Treatment	9.487	<0.001 ***
Location	2.962	0.088 .

Significance codes: “ . ” <0.1, “ * ” <0.05, “ ** “ <0.01, “ *** “ <0.001

Table 8: Pairwise comparison results for enzyme activity based on the one-way ANOVA, using Tukey post hoc test (p<0.05).

Factor	Diff	p adj.
	Site	
Site 2 – Site 1	47.84	0.716
Site 3 – Site 1	-37.94	0.871
Site 4 – Site 1	314.52	<0.001
Site 3 – Site 2	-85.78	0.353
Site 4 – Site 2	266.86	<0.001
Site 4 – Site 3	352.46	<0.001
	Season	
Winter - Autumn	175.58	<0.001
Spring - Autumn	191.35	<0.001
Spring - Winter	15.78	0.942
	Treatment	
NT – LI	-325.69	<0.001
PI – LI	-164.60	0.055
T – LI	-249.90	<0.001
PI – NT	161.09	0.010
T – NT	75.79	0.399
T – PI	-85.30	0.273
	Location	
Rows - Canopy	-69.06	0.088

Soil functions, however, do not occur in isolation, and the evaluation of MBC as influenced by the interaction among the studied factors could provide a comprehensive picture of the response to the transition of conventional to conservation management practices in the studied agroecosystems. This integrated approach enables the identification of agronomic management practices that enhance microbial activity and potentially soil fertility. The combined influence of coupled study region and season demonstrate site-specific response to seasonality with two distinct patterns (Fig. 21). In Site 1 and Site 3, MBC increases over the seasons, reaching highest values during spring. In contrast, the highest MBC values for Site 2 and Site 4 were recorded during winter sampling, with spring exhibiting the second highest values. Notably, the seasonal results of Site 4 were statistically significant compared to the rest of experimental orchards.

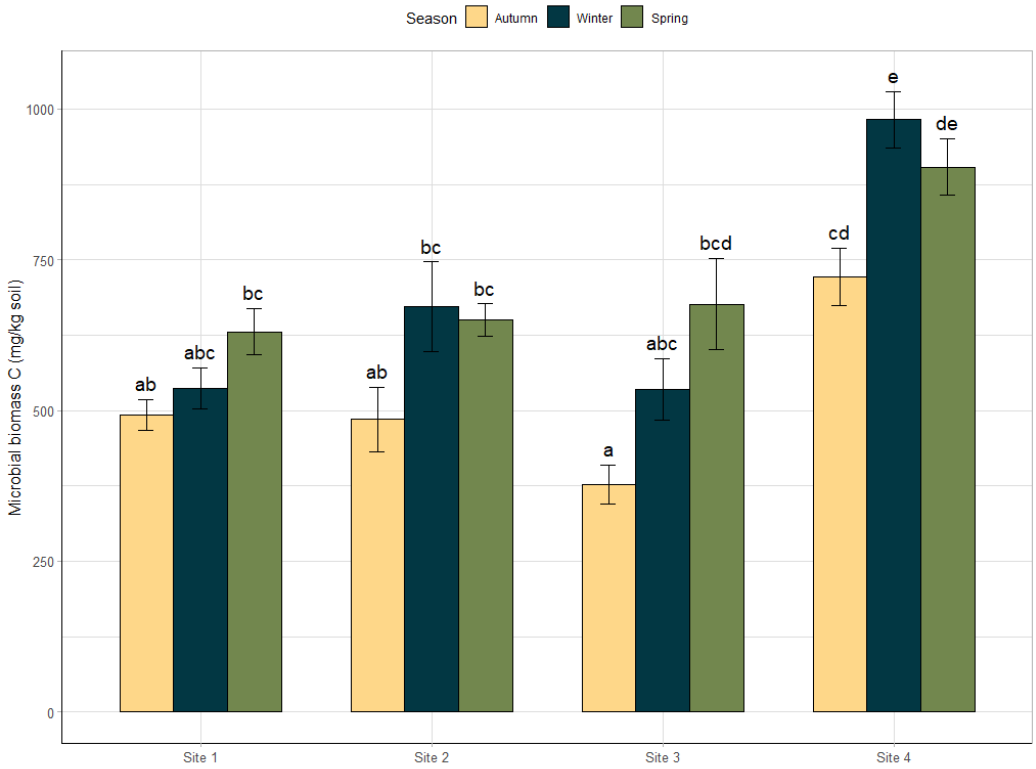

Figure 21: Seasonal variation of microbial biomass C for each of the experimental fields. Error bars represent the standard error. Different lowercase letters indicate statistical significance based on Tukey's post hoc test ($p<0.05$).

The seasonality of SMBC under conventional and conservation management practices is showcased in Figure 22. The results clearly show that conservation practices with organic input (PI and LI) exhibited higher MBC levels than the no-till and tilled treatments across seasons. Moreover, two distinct patters were observed, where

MBC under LI and T treatments increased from autumn to winter and slightly decreased from winter to spring, whereas under NT and PI treatments MBC continued to gradually with each passing season.

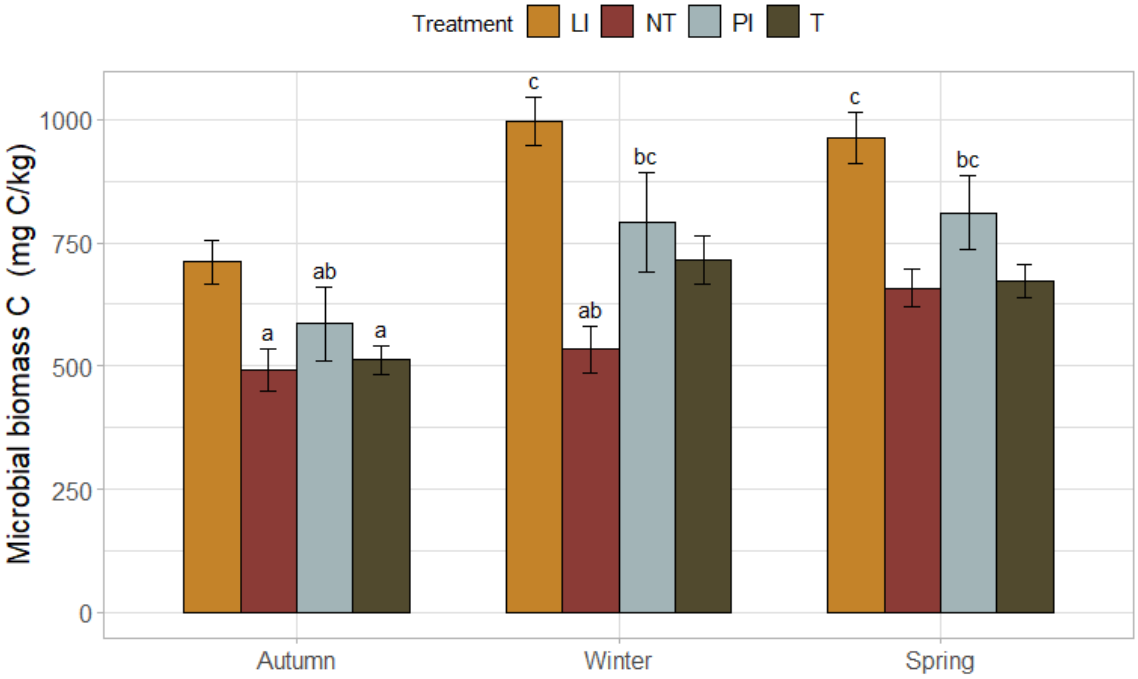

Figure 22: Seasonal soil microbial biomass C under different agronomic management practices. Error bars represent the standard error. Different lower-case letters indicate statistical significance based on Tukey's post-hoc test ($p<0.05$).

Assessing how different management practices interact with soil microbial communities is benefiting the determination of which practices enhance microbial activity. Regarding experimental Site 1, although MBC followed the same seasonal pattern under both management practices, the conservation treatment resulted in slightly higher soil MBC (Fig. 23). A similar effect was observed in Site 4, where higher MBC values were recorded under conservation treatments (LI and PI), reaching highest during winter (995.28 mg C/kg soil and 1100.00 mg C/kg soil, respectively), indicating a different seasonal pattern and the influence of moisture on biological activity. Altogether, the highest values were reported in PI treatment of Site 4, independent of season.

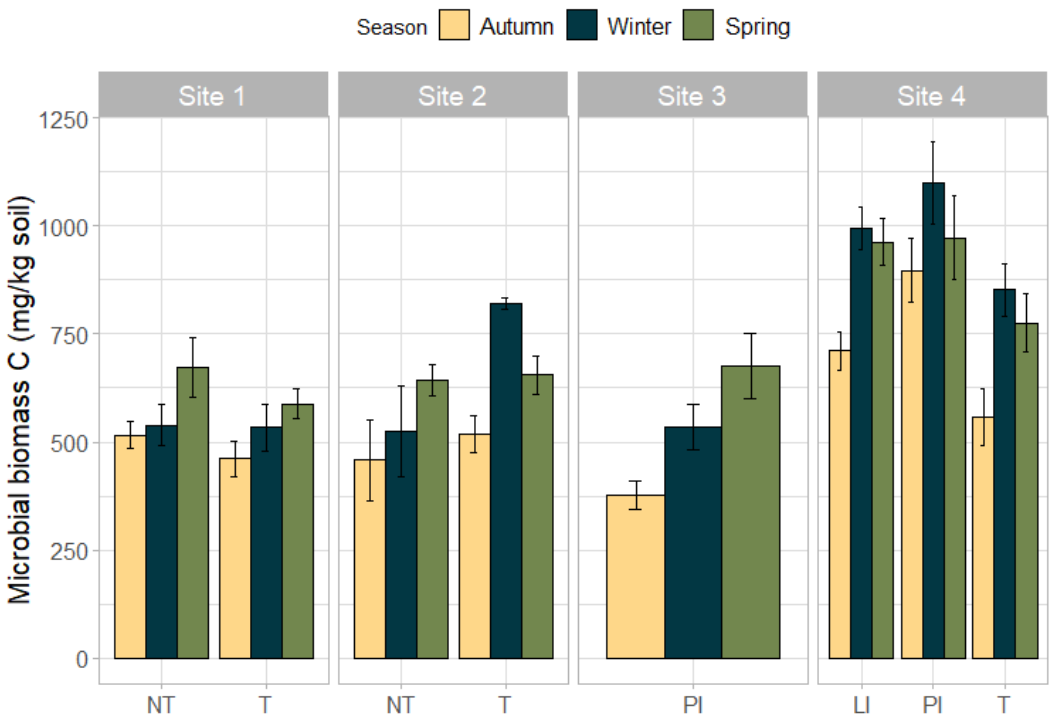

Figure 23: Average microbial biomass C across experimental fields under different agronomic management practices for three consecutive seasons. Error bars represent standard error.

The seasonal relationship between MBC and SOC was evaluated through Pearson's correlation. The results demonstrate that MBC and SOC have a strong positive correlation for both autumn (R=0.78) and winter (R=0.77), reinforcing the ecological link between SOC and microbial biomass (Fig. 24) (Ding et al., 2024). On a regional scale, the experimental sites in Central Crete show slightly stronger correlation during autumn, contrary to results of experimental sites in West Crete (Fig. 25).

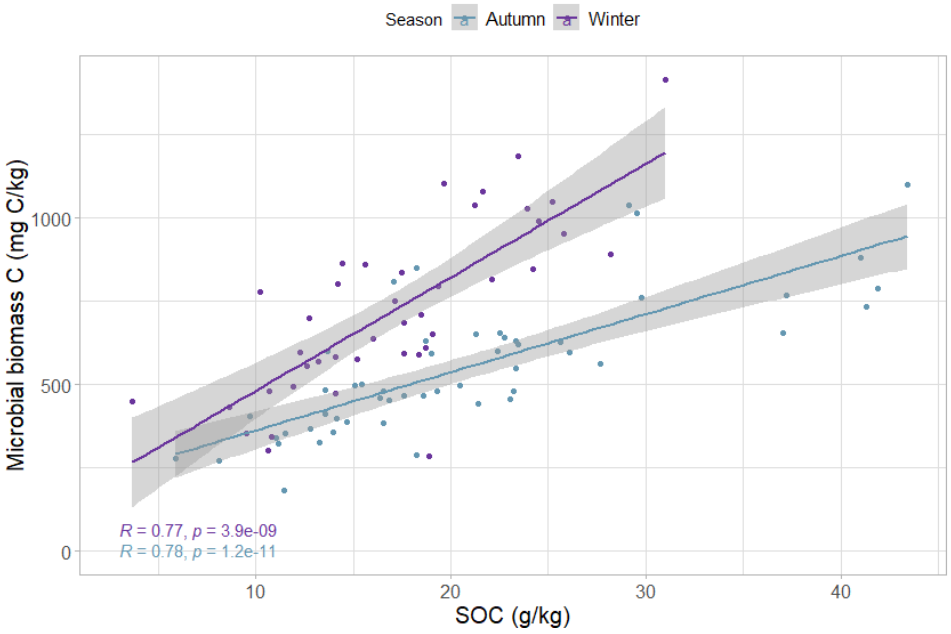

Figure 25: Correlation between microbial biomass C and soil organic C for autumn (blue) and winter (purple).

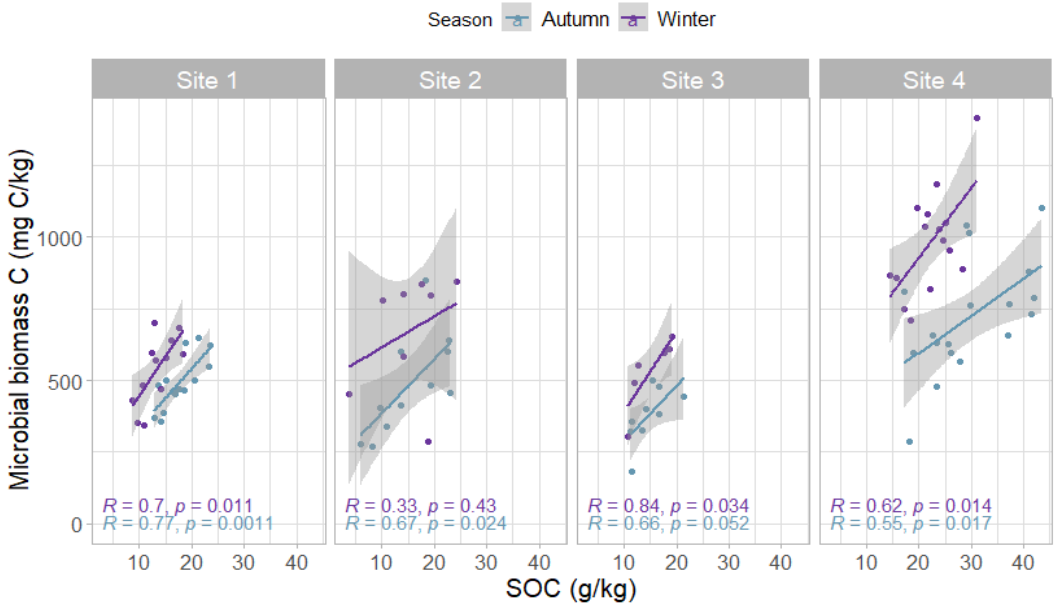

Figure 24: Site-specific correlation between microbial biomass C and soil organic C for autumn (blue) and winter (purple).

The ratio of MBC to SOC is another indicator of soil health, known as soil microbial quotient (SMQ), representing the biologically active fraction of SOM (Ding et al., 2024). Through the monitoring of SMQ, the influence of agronomic management practices on

microbial biomass can be observed in Figure 26. In the experimental Site 4, SMQ was higher under conservation treatments, with SMQ under PI treatment 0.36% higher than LI and 0.8% higher than conventional treatment.

The seasonal pattern of SMQ under different agronomic management practices is similar to MBC, with higher biologically active fraction of SOM during winter (Fig. 27). In the control field (T) of Site 1, SMQ was 0.97% higher than conservation field during winter and 0.26% during autumn.

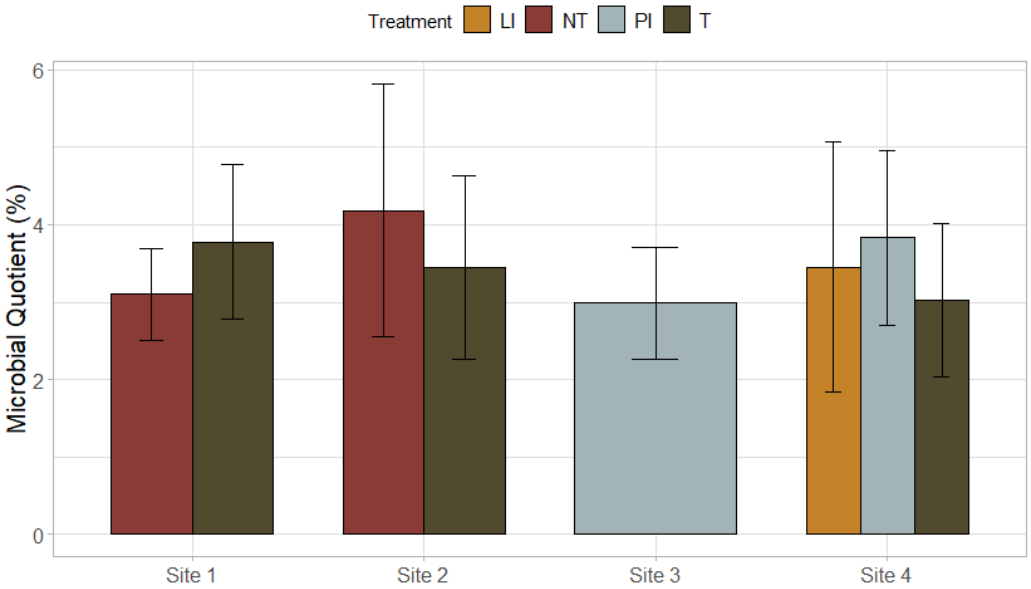

Figure 26: Combined influence of study region and treatment on soil microbial quotient. Error bars indicate standard error.

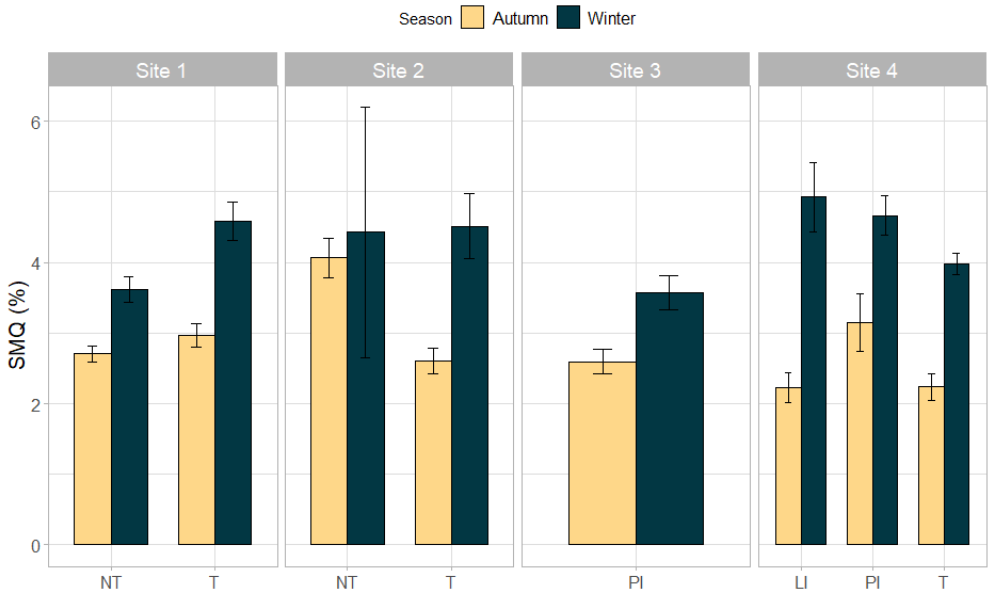

Figure 27: Seasonality, treatment and study region coupled influence on soil microbial quotient. Error bars represent standard error.

3.5 Soil extracellular enzyme activity

To better understand the response of OM decomposers under conservation management across different seasons, extracellular enzyme activity was assessed for the selected experimental orchards. The soil enzyme activities of β-N-acetylhexosaminidase, β-xylosidase, phosphatase, and β-glucosidase were measured for three consecutive season samplings. These EE activities were documented and analysed to ascertain the influence that the selected study region, treatments, seasons and specific locations inside fields (below tree canopy and between rows) have on them.

3.5.1 β-glucosidase

The activity of β-glucosidase for the selected experimental orchards is shown in Figure 28, while the comparison between each of their level is presented in Table 9 highlighting the individual effects of said factors. The comparison was conducted using one-way ANOVA for each factor (Table 10) and Tukey's post hoc test.

Season has the highest influence on BG, with highest observed activity during autumn and winter (164.88 nmol/g soil/h and 168.80 nmol/g soil/h, respectively) compared to spring (107.95 nmol/g soil/h), showcasing significant difference. While ST during autumn and winter was lower than spring, soil moisture was higher resulting in increased mobility of microorganisms, enzymes and nutrients (Zuccarini et al., 2022). In environments with strong seasonality such as the Mediterranean, soil enzymes are especially affected by the heat/drought stress and water scarcity through the reduction of their capacity to contact their specific substrate (Baldrian et al., 2013; Zuccarini et al., 2022). Besides the strong seasonal effect, β-glucosidase activity was affected by the utilized agronomic practices ($p<0.022$). The conservation treatments were overall higher than the conventional, while the highest average activity was under LI (199.42 nmol/g soil/h) agronomic management. Moreover, β-glucosidase showed no significant difference for the study region or location in the field.

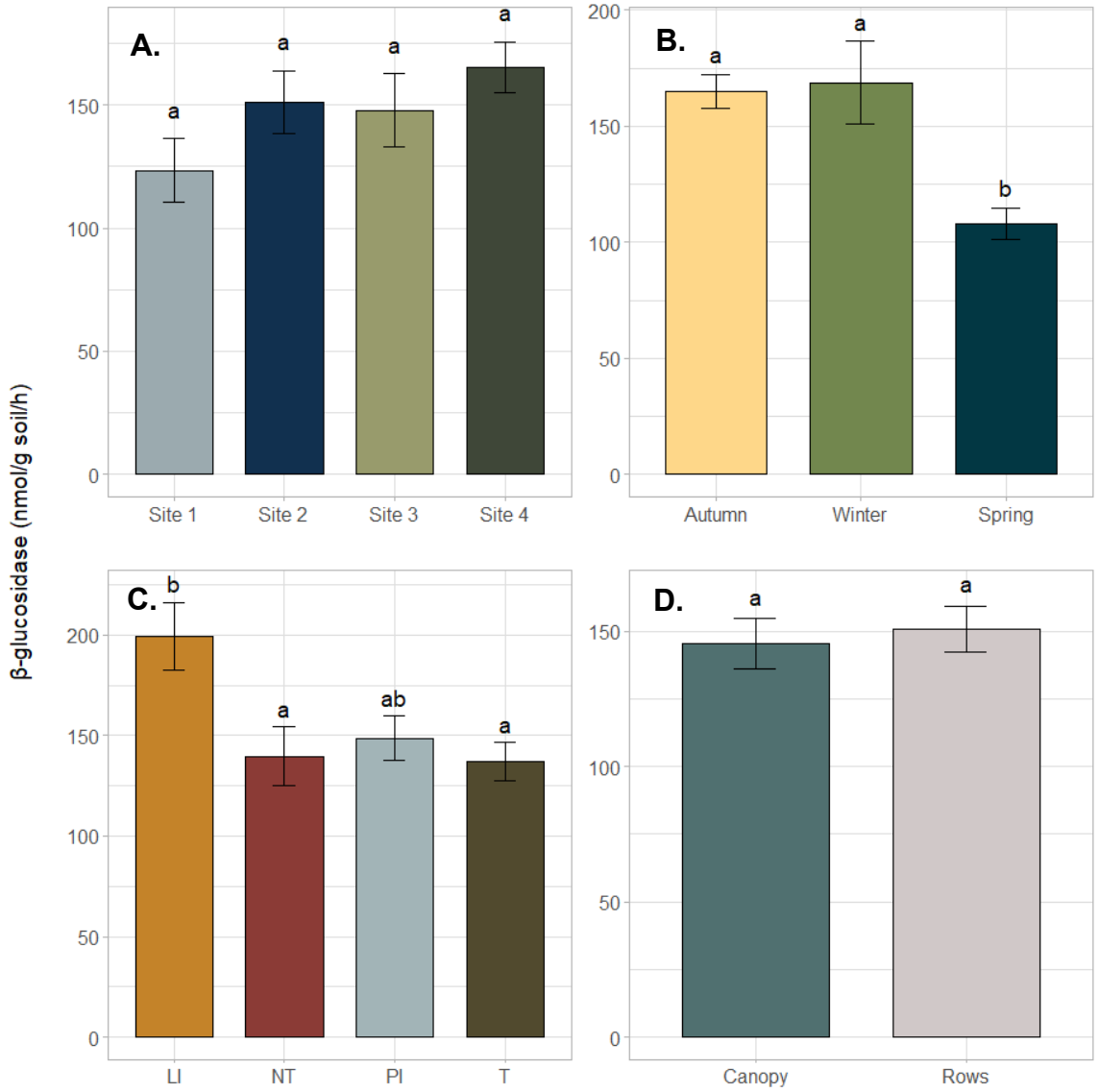

Figure 28: Activity of β-glucosidase across four factors: A. study region, B. season, C. treatment, and D. location—represented in four separate plots. Error bars represent the standard error. Different lower-case letters on top of bars indicate significant difference between the levels of said factor, based on Tukey HSD ($p<0.05$).

Table 9: β-glucosidase activity one-way ANOVA results for each of the studied factors.

Factor	F-value	p-value
Site	2.23	0.086 .
Season	10.11	<0.001 ***
Treatment	3.26	0.022 *
Location	0.19	0.661

Significance codes: “ . ” <0.1, “ * ” <0.05, “ ** “ <0.01, “ *** “ <0.001

Table 10: Pairwise comparison results for β-glucosidase activity based on the one-way ANOVA using Tukey HSD test (p<0.05).

Factor	Diff	p adj.
	Site	
Site 2 – Site 1	27.64	0.399
Site 3 – Site 1	24.41	0.599
Site 4 – Site 1	41.73	0.052
Site 3 – Site 2	-3.23	0.998
Site 4 – Site 2	14.09	0.829
Site 4 – Site 3	17.31	0.790
	Season	
Winter - Autumn	3.93	0.963
Spring - Autumn	-56.93	0.0002
Spring - Winter	-60.86	0.0005
	Treatment	
NT – LI	-59.66	0.035
PI – LI	-50.90	0.084
T – LI	-62.50	0.015
PI – NT	8.76	0.956
T – NT	-2.85	0.998
T – PI	-11.60	0.877
	Location	
Rows - Canopy	5.50	0.661

To gain insights on the influence of the interaction of the abovementioned factors, multi-way ANOVA was applied (Table 11). Apart from Site 3, the activity of BG increased from autumn to winter, while it decreased from winter to spring for all experimental sites (Fig. 29). Apart from Site 3, the trend of BG follows the abovementioned trend of SOC and MBC during autumn and winter. The highest variation between autumn and winter occurred in Site 4 with detected winter activity being 19.26 nmol/g/h higher than the autumn. These variations while distinct, they were not statistically significant different. During spring, BG activity reached lowest across sampling sites, while the lowest was detected in Site 1 (47.32 nmol/h/g).

Table 11: Multi-way ANOVA of the interactions of the studied factors on enzymatic activity.

Interactions	F-value	p-value
Site – Season	3.695	<0.001 ***
Site - Treatment	2.221	0.033 *
Season - Treatment	4.364	<0.001 ***
Site – Season – Treatment	3.207	< 0.001 ***
Site – Season – Treatment - Location	1.918	0.009 **

Significance codes: “ . ” <0.1, “ * ” <0.05, “ ** “ <0.01, “ *** “ <0.001

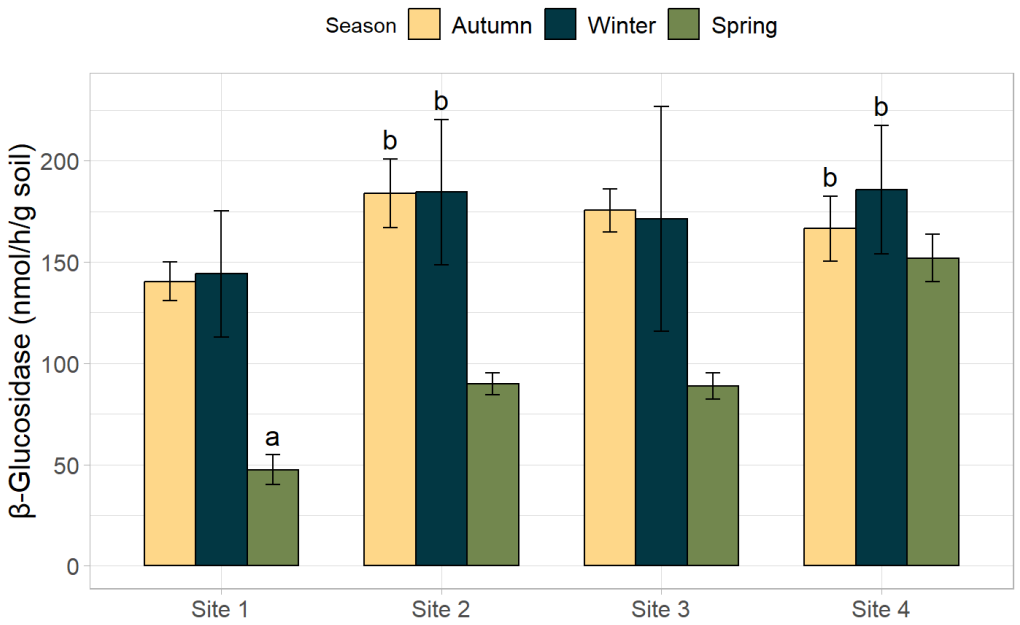

Figure 29: Average β-glucosidase activity of the experimental sites for autumn (yellow), winter (blue) and spring (green). Error bars represent the standard error for each Site and Season. Different lower-case letters on top of bars indicate significant difference based on Tukey HSD ($p<0.01$)

It is worth noting that, based on the observed BG activity across seasons and under different agronomic practices, no clear conclusions could be reached. As demonstrated in Figure 30, during spring, BG activity was highest under LI (229.59 nmol/h/g) and lowest under conventional tillage (142.72 nmol/h/g). On the other hand, conventional tillage had the overall highest BG activity during winter reaching 187.96 nmol/h/g while NT had the lowest with 132.35 nmol/h/g. During spring, BG activity was once again highest under LI (191.14 nmol/h/g) while lowest under NT (68.30 nmol/h/g).

When investigating the site-specific effect of seasonality and management practices, we observe that the overall highest BG activity was detected in Site 2 during winter for the conventional treatment (254.41 nmol/h/g) and the overall lowest activity in the conservation treatment of Site 1 during spring (55.33 nmol/h/g) (Fig. 31). Besides PI and NT treatments in Site 4, the activity measured during autumn and winter were higher independent of treatment, across all cases.

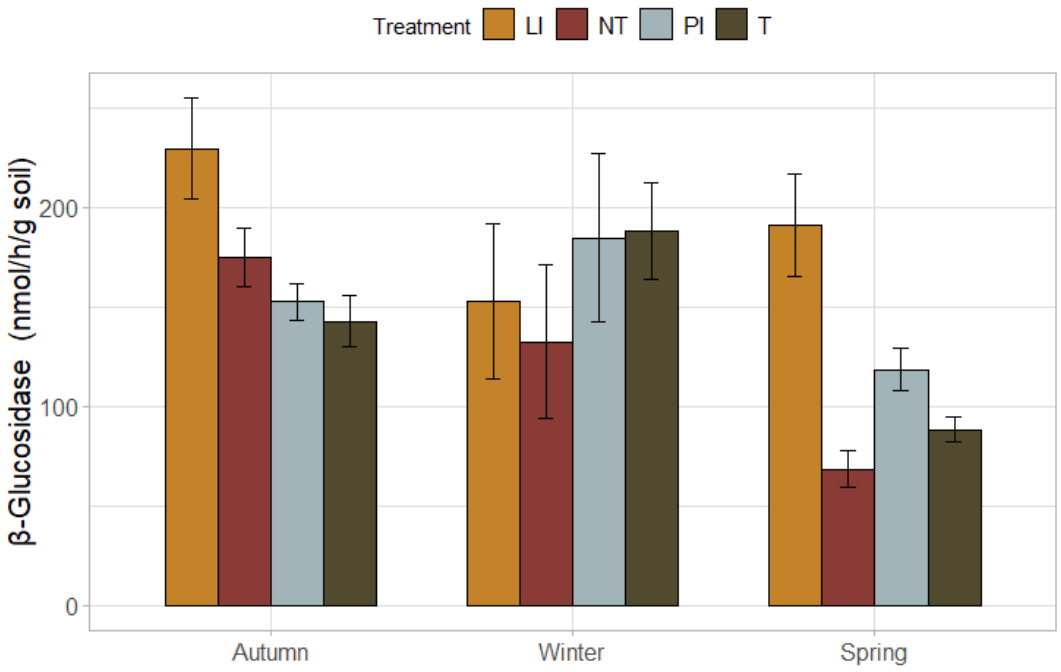

Figure 31: Seasonality and agronomic management practice coupled effect on β-glucosidase activity. Error bars represent standard error.

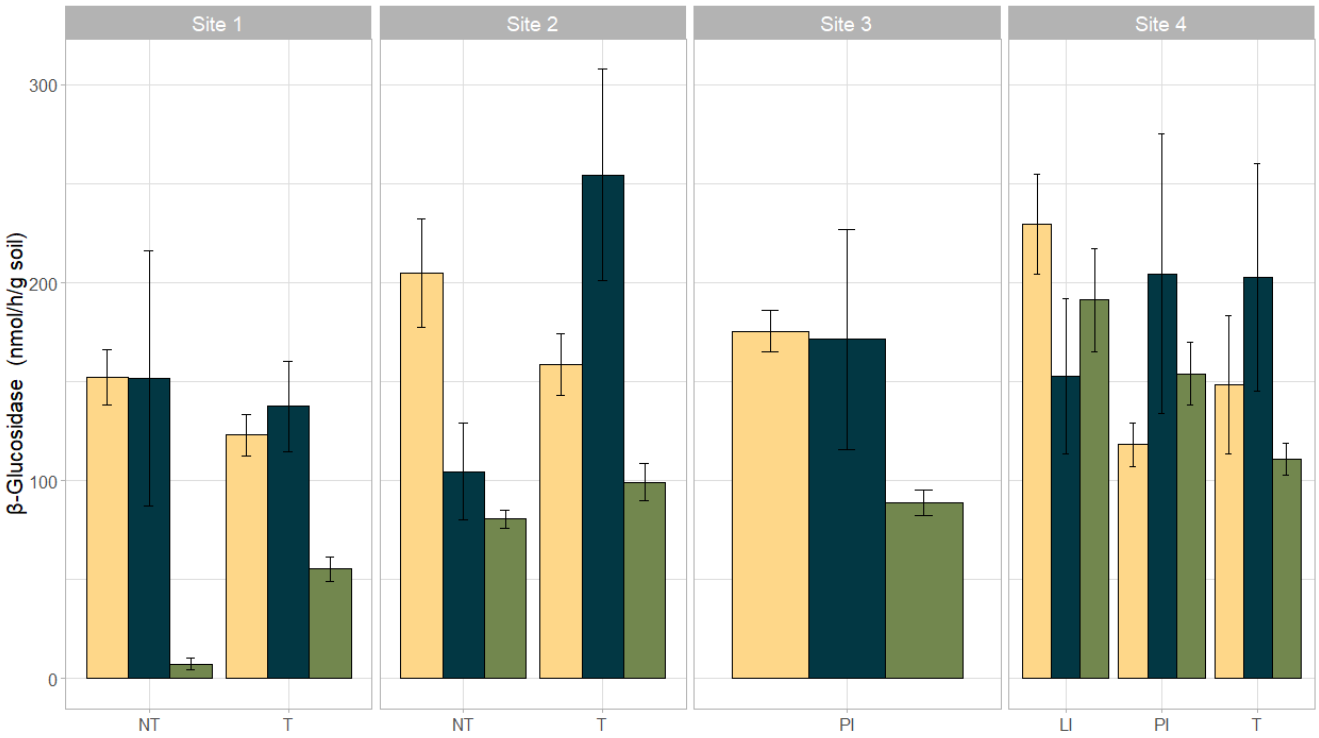

Figure 30: Average β-glucosidase activity of the experimental sites for autumn (yellow), winter (blue) and spring (green). Error bars represent the standard error for each site and season.

Pearson's correlation was utilised to assess the seasonal correlation of BG under different agronomic management practices and soil moisture content. During winter, no correlation was detected between SM and BG under conservation treatments, while a moderate negative correlation can be observed for the conventional treatment (R=-0.58, p=0.031) (Fig. 32). In the case of spring, strong correlations were detected for NT (R=0.87, p=0.011), PI (R=0.72, p=0.012) and T (R=0.78, p<0.001).

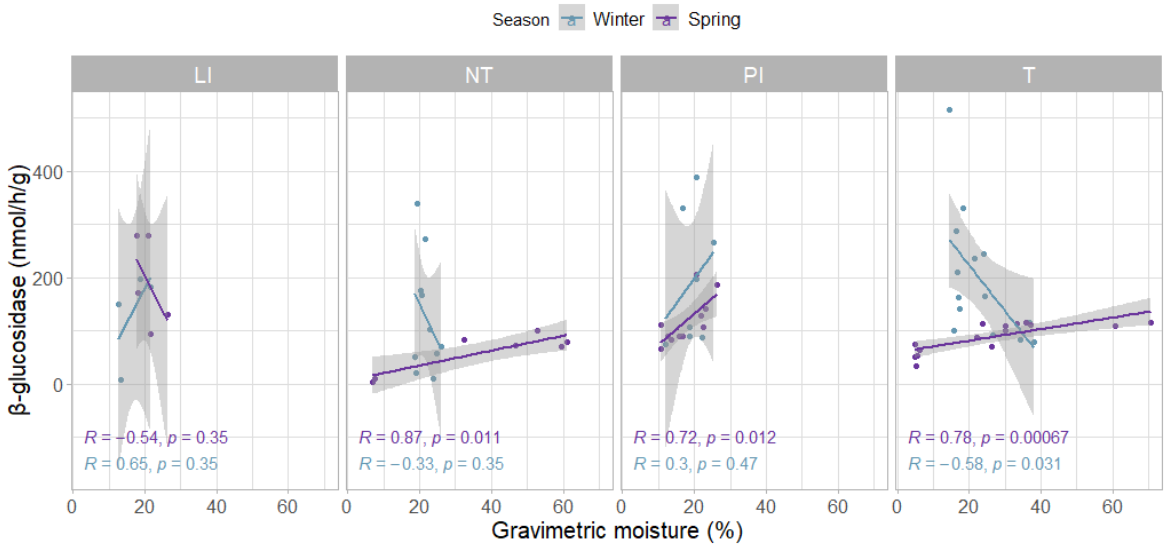

Figure 32: Pearson's correlation between β-glucosidase and soil moisture content for winter (light blue) and spring (purple).

When the seasonal correlation between BG and SOC was evaluated through Pearson's correlation, the results demonstrated no significant correlation between them (R=0.012 for autumn and R=0.2 for winter) (Fig. 33). When seasonal correlation between BG and SOC was investigated regionally, results revealed that they were slightly positively correlated in Site 1 during autumn and Site 2 during winter while negatively correlated during winter in Site 3 (Fig. 34). In Site 4, no correlation was detected overall.

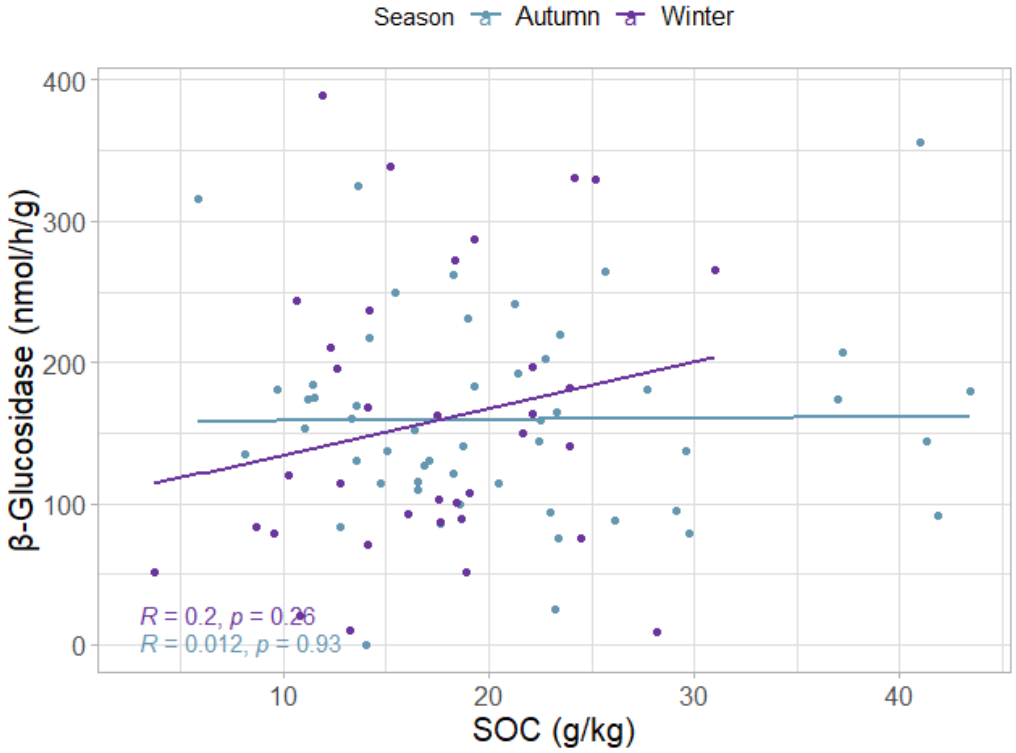

Figure 33: Pearson's correlation between β-glucosidase and SOC for autumn (light blue) and winter (purple).

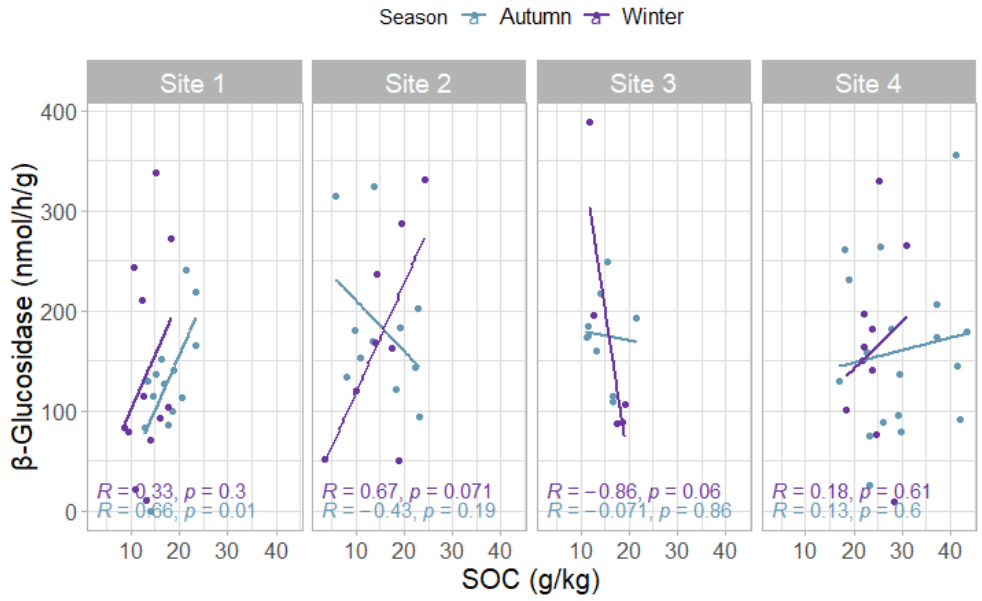

Figure 34: Pearson's correlation between β-glucosidase and SOC for autumn (light blue) and winter (purple) at each experimental site.

3.5.2 β-Xylosidase

The activity of XYL for the selected experimental orchards is presented in Figure 35. Similar to BG, the comparison was conducted using one-way ANOVA for each factor (Table 12) and Tukey's post hoc test (Table 13).

Study region significantly influenced XYL activity, with highest average observed activity in Site 2 (66.92 nmol/h/g) and contrary to BG, lowest average activity was detected in Site 4 (27.55 nmol/h/g). These differences were statistically significant based on Tukey's test ($p<0.020$). Beyond regional differences, seasonality had the most profound impact on XYL activity, with the highest average activity during winter (80.34 nmol/h/g) and reached lowest average during spring (18.38 nmol/h/g). In contrast, average XYL activity did not show a clear pattern concerning agronomic management practices or sampling location. Although some variations were noted, these differences were not statistically significant.

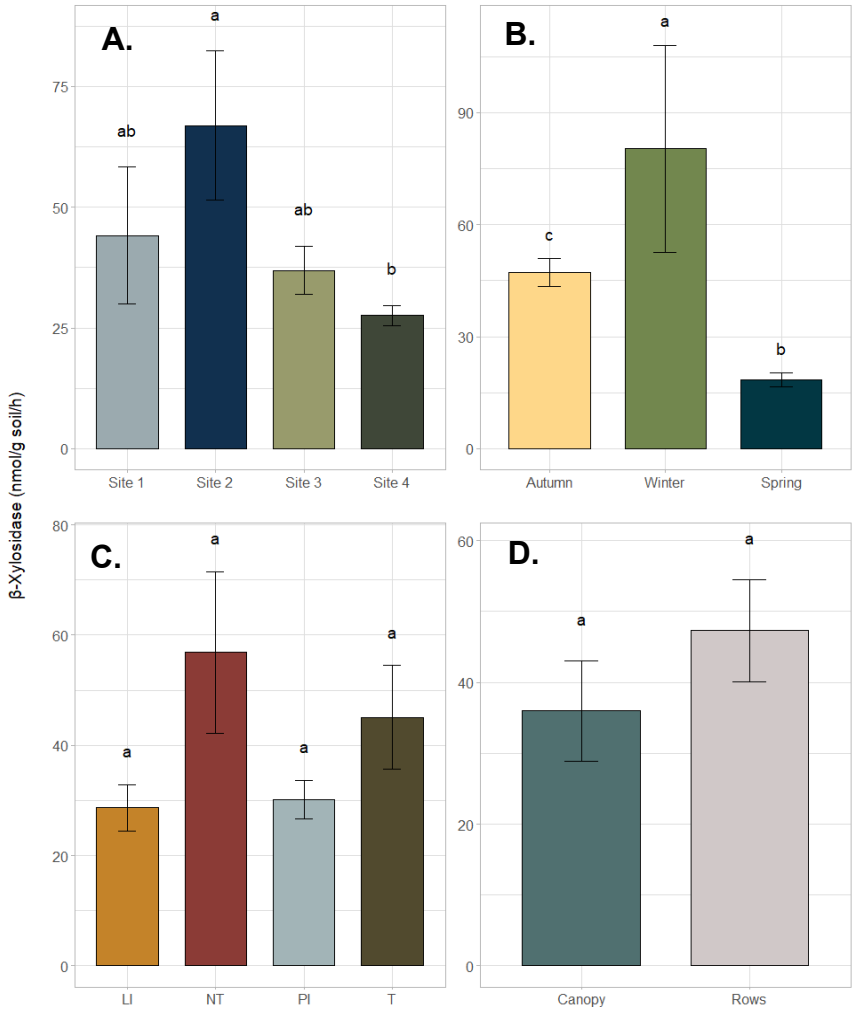

Figure 35: Activity of β-xylosidase across four factors: A. study region, B. season, C. treatment, and D. location. Error bars represent the standard error for each factor at the respective plot. Different lower-case letters on top of bars indicate significant difference between the levels of said factor, based on Tukey HSD ($p<0.05$).

Table 12: β-xylosidase activity one-way ANOVA results for each of the studied factors.

Factor	F-value	p-value
Site	2.193	0.036 *
Season	9.645	<0.001 ***
Treatment	1.545	0.204
Location	1.239	0.267

Significance codes: " . " <0.1, " * " <0.05, " ** " <0.01, " *** " <0.001

Table 13: Pairwise comparison results for β-xylosidase activity based on the one-way using Tukey HSD test (p<0.05).

Factor	Diff	p adj.
	Site	
Site 2 – Site 1	22.79	0.409
Site 3 – Site 1	-7.20	0.969
Site 4 – Site 1	-16.58	0.584
Site 3 – Site 2	-29.99	0.252
Site 4 – Site 2	-39.37	0.020
Site 4 – Site 3	-9.38	0.920
	Season	
Winter - Autumn	33.23	0.049
Spring - Autumn	-28.71	0.022
Spring - Winter	-61.95	<0.001
	Treatment	
NT – LI	28.13	0.389
PI – LI	1.48	0.999
T – LI	16.39	0.767
PI – NT	-26.64	0.225
T – NT	-11.73	0.811
T – PI	14.91	0.651
	Location	
Rows - Canopy	11.31	0.267

NT = no-till; LI = no-till with pruning's incorporation and legumes intercropping; PI = no-till with pruning's incorporation; T = mechanical plough.

To further investigate the effect of the combined interactions of the studied factors on XYL activities, multi-way ANOVA was conducted. The analysis revealed that the examined coupled factors were of statistical significance (Table 14), highlighting the sensitivity of XYL activity, apart from the interactions between study region and management practices (p=0.11). As illustrated in Figure 36, a regional trend emerged between sampling sites of West and Central Crete. The experimental orchards in Central Crete (Site 1 and Site 2) exhibited significantly higher XYL activities (356.69 nmol/g soil/h and 239.65 nmol/g soil/h, respectively), while the average XYL activities

of the ones in West Crete (Site 3 and Site 4) were highest during autumn with values 56.89 nmol/h/g and 32.96 nmol/h/g, respectively. It is worth mentioning that in Site 1 and Site 2, XYL activities follow similar pattern to BG activities while being statistically significant to the rest of sites and seasons, but not between them.

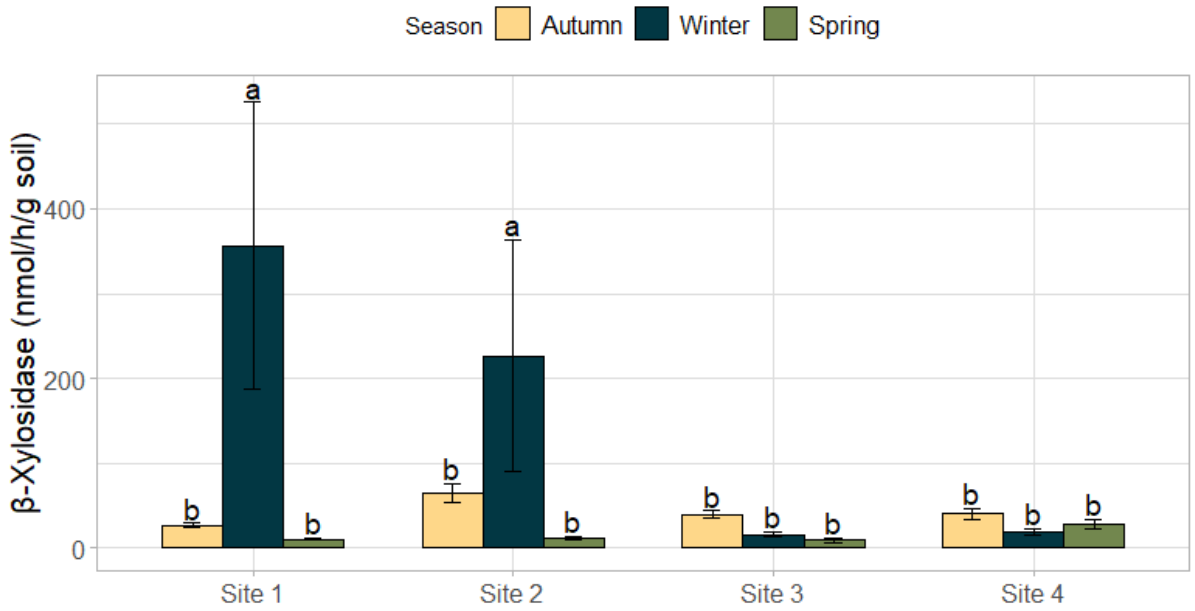

Figure 36: Average β-xylosidase activity of the experimental sites for autumn (yellow), winter (blue) and spring (green). Error bars represent the standard error for each Site and Season. Different lower-case letters on top of bars indicate significant difference for the specific interaction of Site and Season, based on Tukey HSD ($p<0.001$).

Table 14: β-Xylosidase activity multi-way ANOVA results based on the interactions of the studied factors.

Interactions	F-value	p-value
Site – Season	19.69	<0.001 ***
Site - Treatment	1.70	0.110
Season - Treatment	9.93	<0.001 ***
Site – Season – Treatment	16.96	<0.001 ***
Site – Season – Treatment - Location	11.89	<0.001 ***

Significance codes: “ . ” <0.1, “ * ” <0.05, “ ** “ <0.01, “ *** “ <0.001

When evaluating the coupled effect of seasonality and management practices, results revealed that conservation treatment LI had the lowest average XYL activity during autumn (35.32 nmol/h/g) while the rest of the treatments had slightly higher activities (48.01 nmol/h/g - 48.70 nmol/h/g). During winter, conservation treatments LI and PI had lower activities (11.28 nmol/h/g and 13.71 nmol/h/g, respectively) than NT (281.81 nmol/h/g) and T (107.65 nmol/h/g), while NT being significantly different (Fig. 37).

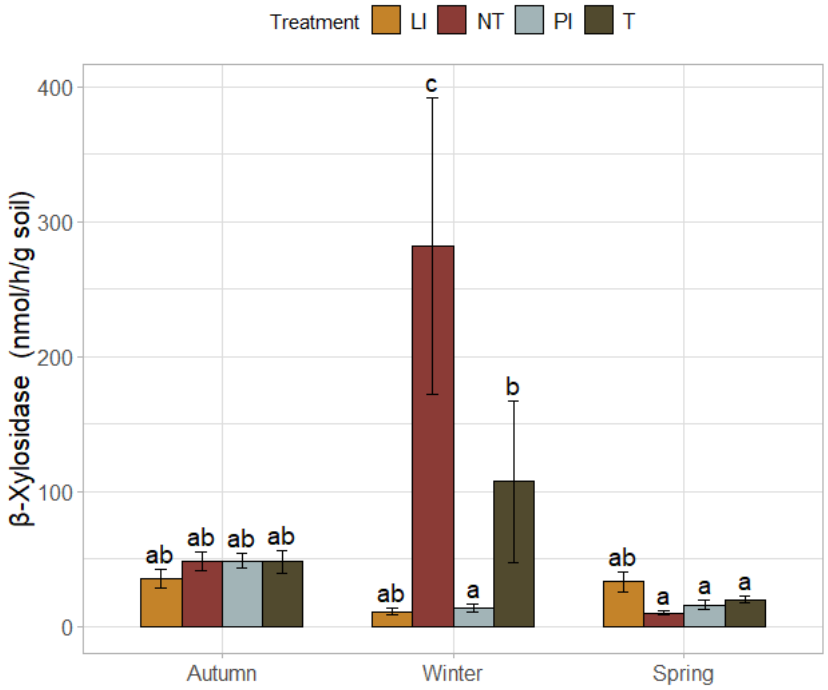

Figure 37: Average β-xylosidase activity across seasons under different agronomic management (colour). Error bars represent standard error. Different lower-case letters on top of bars indicate significant difference for the specific interaction of Site and Season, based on Tukey HSD ($p<0.001$).

A clear seasonal pattern is evident between Central and West Crete, across experimental sites and management practices (Fig. 38). Specifically, average XYL activities during winter showed strong contrast between C. and W. Crete, with highest XYL activities recorded in C. Crete (486.24 nmol/h/g under NT in Site 1 and 380 under T in Site 2) and lowest activities in W. Crete (15.31 nmol/h/g under PI in Site 3 and 11.28 nmol/h/g under LI in Site 4). In C. Crete, the lowest XYL activities occurred during spring, regardless of management practice, whereas in W. Crete, the highest activity was observed during autumn.

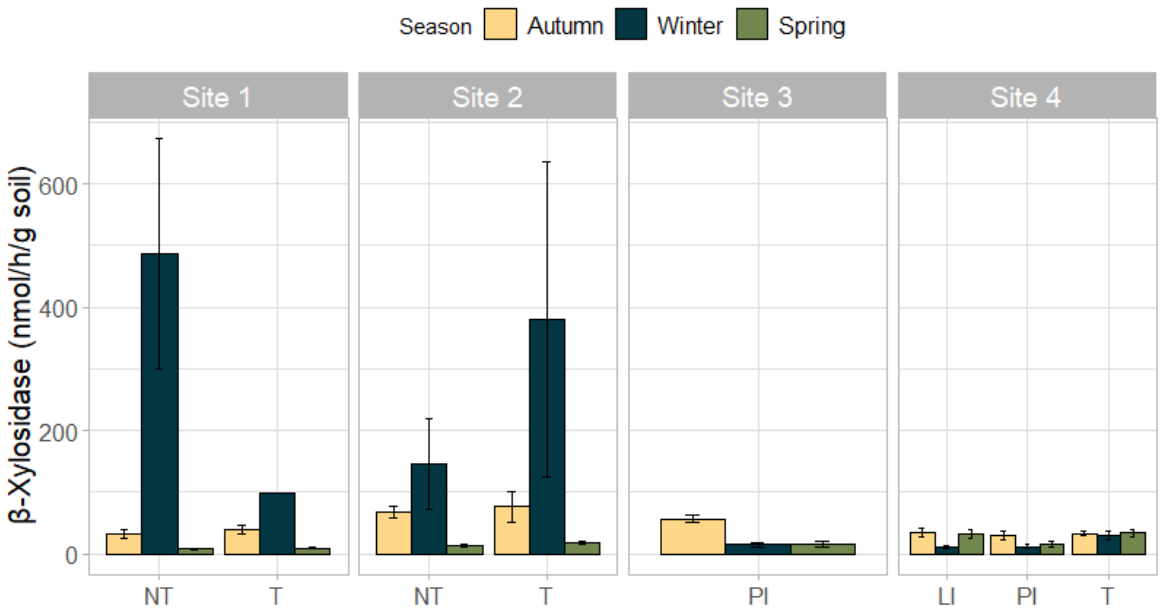

Figure 38: Average β-xylosidase activity across seasons and experimental sites under different agronomic management. Error bars represent standard error.

3.5.3 Phosphatase

Phosphatase activity was significantly influence by the study area and seasonality, without taking their interactions into consideration (Table 15). Based on the results, Site 2 had the highest overall average PH activity (602 nmol/h/g), while the lowest detected PH activity was 360 nmol/h/g (Site 1) (Fig. 39), and the difference between the two sites was statistically significant (p adj. <0.001) (Table 16). Regarding the seasonal effect on PH activity, winter values were significantly higher than those in autumn and spring, with average activities of 687 nmol/h/g, 385 nmol/h/g and 346.84 nmol/h/g respectively. Concerning the effect of treatment, LI had the lowest results (320 nmol/h/g) while NT had the highest, but no significant differences were detected (Table 16). No variation was observed between samples collected from under the tree canopy and those from the inter-row area.

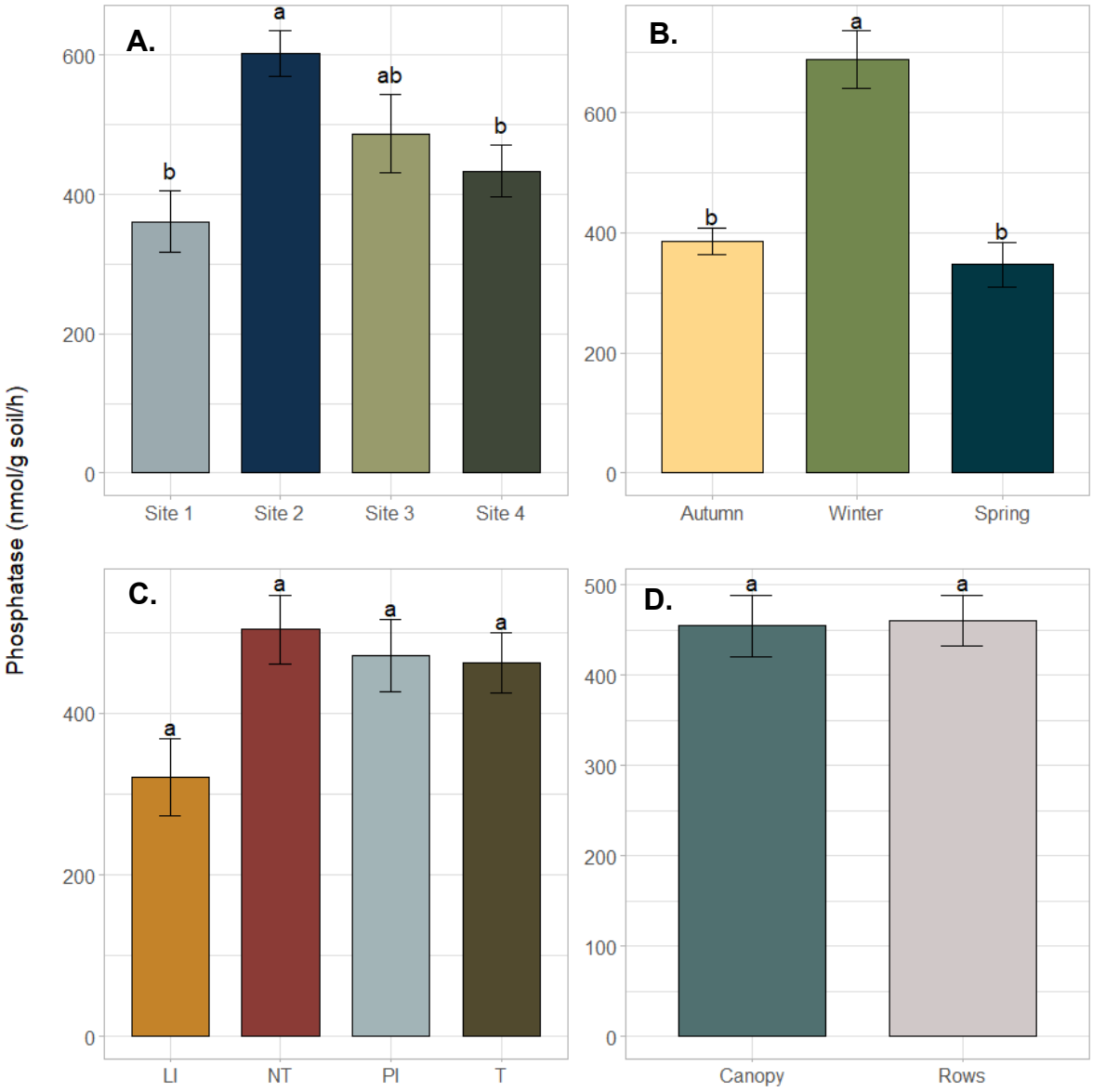

Figure 39: Average phosphatase activity based on four factors: A. study region, B. season, C. treatment, and D. location—represented in four separate plots. Each plot shows the variation in enzyme activity with respect to one factor independently. Error bars represent the standard error for each factor at the respective plot. Different lower-case letters on top of bars indicate significant difference between the levels of said factor, based on Tukey HSD ($p<0.05$).

Table 15: Phosphatase activity one-way ANOVA results for each of the studied factors.

Factor	F-value	p-value
Site	5.70	<0.001 ***
Season	26.16	<0.001 ***
Treatment	2.09	0.102
Location	0.02	0.896

Significance codes: “ . ” <0.1, “ * ” <0.05, “ ** “ <0.01, “ *** “ <0.001

Table 16: Pairwise comparison results for phosphatase activity based on the one-way ANOVA, using Tukey HSD test (p<0.05).

Factor	Diff	p adj.
	Site	
Site 2 – Site 1	241.59	<0.001
Site 3 – Site 1	126.82	0.306
Site 4 – Site 1	72.99	0.513
Site 3 – Site 2	-114.78	0.428
Site 4 – Site 2	-168.60	0.016
Site 4 – Site 3	-53.82	0.69
	Season	
Winter - Autumn	302.32	<0.001
Spring - Autumn	-38.80	0.69
Spring - Winter	-341.13	<0.001
	Treatment	
NT – LI	182.69	0.070
PI – LI	150.01	0.195
T – LI	141.63	0.190
PI – NT	-32.68	0.950
T – NT	-41.06	0.879
T – PI	-8.38	0.999
	Location	
Rows - Canopy	5.70	0.896

NT = no-till; LI = no-till with pruning’s incorporation and legumes intercropping; PI = no-till with pruning’s incorporation; T = mechanical plough.

By exploring the interactions of abovementioned factors on PH activity, we observe the significant influence of the factor's interaction (Table 17). The interaction between study region and season appear to have the strongest impact on the activity, contrary to the interaction of site and treatment, which has the least effect, though still significant.

The seasonal effect on PH activity at a regional scale is presented in Figure 40. The highest average PH activity was detected during winter across the experimental fields, besides Site 3. In Site 1, the average PH activities during autumn, winter and spring were 292.58 nmol/h/g, 744.42 nmol/h/g, and 33.96 nmol/h/g, respectively. In the case of Site 2, average PH activity was highest during winter (814.58 nmol/h/g), while autumn and spring showed nearly identical levels (531.37 nmol/h/g and 531.71 nmol/g/h). Contrary to Site 1, in Site 4 while highest activity was observed during winter (683.77), PH reached second-highest activity in autumn with lowest activity in spring (473.16 nmol/h/g and 213.23 nmol/h/g, respectively). Site 3 deviated from this trend, showing highest PH activity during autumn (697.09 nmol/h/g) and the lowest, similar to Site 1, in spring (141.92 nmol/h/g).

Table 17: Phosphatase activity multi-way ANOVA results for interactions of the studied factors.

Interactions	F-value	p-value
Site – Season	19.03	<0.001 ***
Site - Treatment	3.649	<0.001 ***
Season - Treatment	9.06	<0.001 ***
Site – Season – Treatment	12.34	<0.001 ***
Site – Season – Treatment - Location	8.06	<0.001 ***

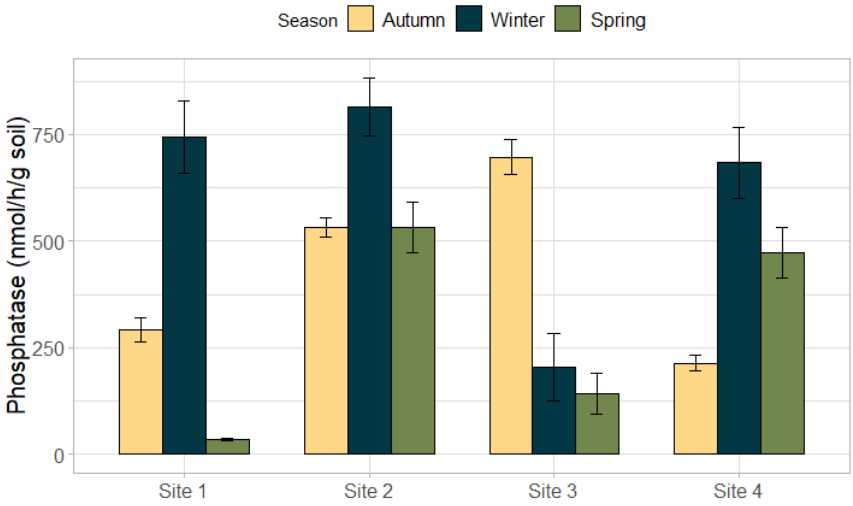

Figure 40: Average seasonal phosphatase activity across experimental sites. Error bars represent standard error.

When the interactions of seasonality and agronomic management are considered, overall PH activity increases from autumn to winter and then decreases from winter to spring for all treatments besides PI (Fig. 41). During autumn, PI exhibited highest average PH activity (532.56 nmol/h/g), while LI the lowest (212.23 nmol/h/g). In winter, the highest average PH activity was observed under NT (842.45 nmol/h/g) and the lowest under PI (500.56 nmol/h/g). In spring, PH was highest under the conventional treatment (461.34 nmol/h/g) and lowest under LI (187.15 nmol/h/g).

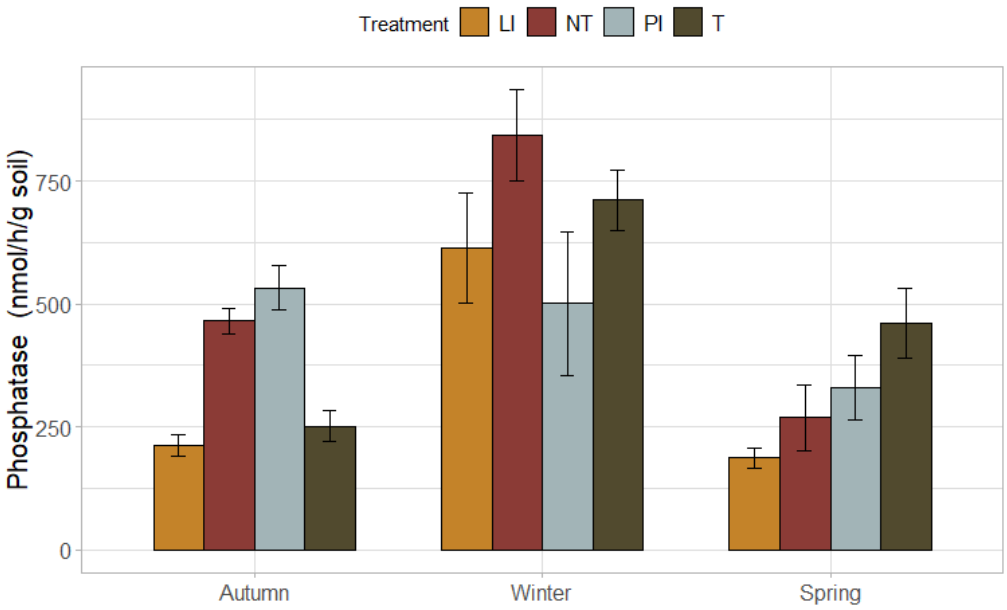

Figure 41: Average seasonal phosphatase activity under different agronomic management practices. Error bars represent standard error.

When the interactions of study region, season and treatment are examined, a clear trend on Site 1 can be perceived, with highest and lowest activity during winter and spring, respectively (Fig. 42). Additionally, only the activity under conventional treatment (T) of Site 4 was higher during spring sampling (790.87 nmol/h/g). Aside from Site 3 and conventional treatment of Site 4, all the other interactions between study region and management practice were highest during winter sampling.

The seasonal relationship between PH and SM was evaluated using Pearson's correlation. According to the results, no significant correlation was detected between PH and SM during winter ($R=0.22$, $p=0.17$), whereas a strong correlation was observed for spring ($R=0.61$, $p<0.001$) (Fig. 43).

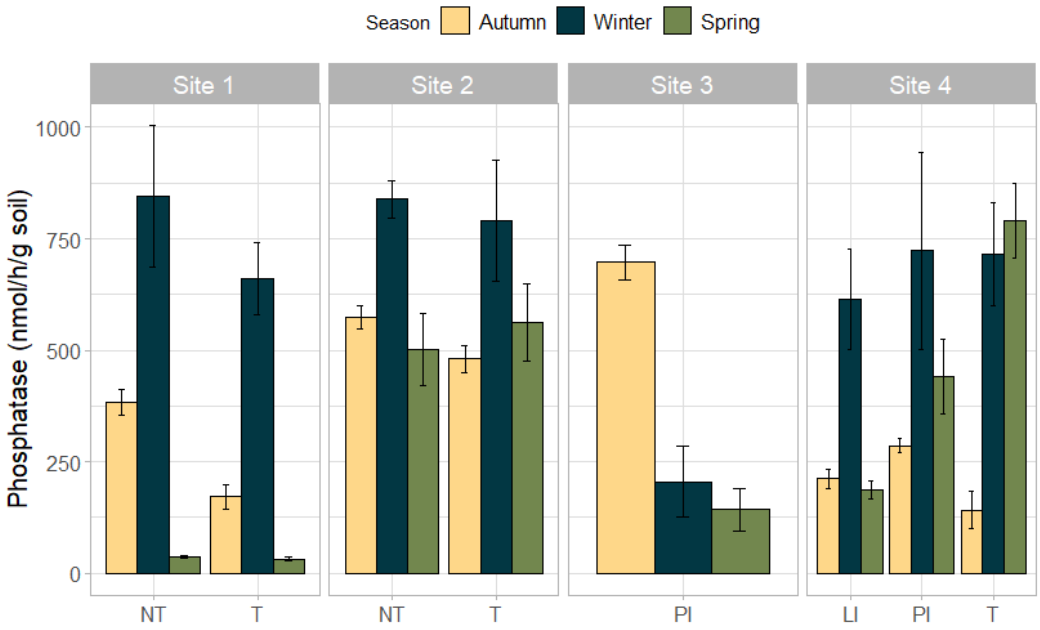

Figure 42: Seasonality of phosphatase activity across experimental sites under different agronomic treatments. Error bars represent standard error.

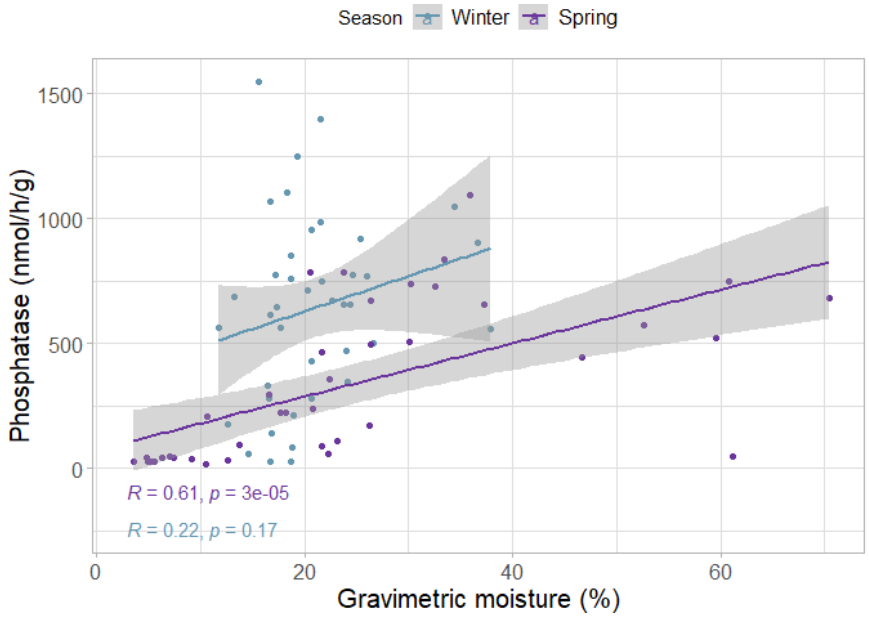

Figure 43: Correlation between phosphatase activity and soil moisture content for winter (blue) and spring (purple).

3.5.4 β-N-acetyl-glucosaminidase

The activity of extracellular NAG was primarily influenced by study region (p=0.001) and seasonality (p<0.001), individually, while treatment and location had no effect on it (p=0.49 and p=0.12, respectively; Table 18). Specifically, the average activity at Site 1 (58.63 nmol/h/g) was nearly twice as high and statistically different from the activities of the rest of experimental orchards (Site 2: 24.67 nmol/h/g; Site 3: 28.98 nmol/h/g; Site 4: 29.14 nmol/h/g) (Fig. 44; Table 19). Seasonality appears to favour NAG activity during winter (94.95 nmol/h/g), which is over three times greater than autumn (29.70 nmol/h/g) and spring (17.97 nmol/h/g). Regarding management practices, the average activity of NT (43.90 nmol/g soil/h) and T (40.38 nmol/h/g) were slightly higher compared to LI (35.20 nmol/h/g) and PI (29.29 nmol/h/g), though the differences were not statistically significant. Similarly to management practices, location had no significant effect on NAG activity, as the difference between samples taken between the rows and below the canopy was only 11 nmol/h/g and not statistically significant (p=0.12).

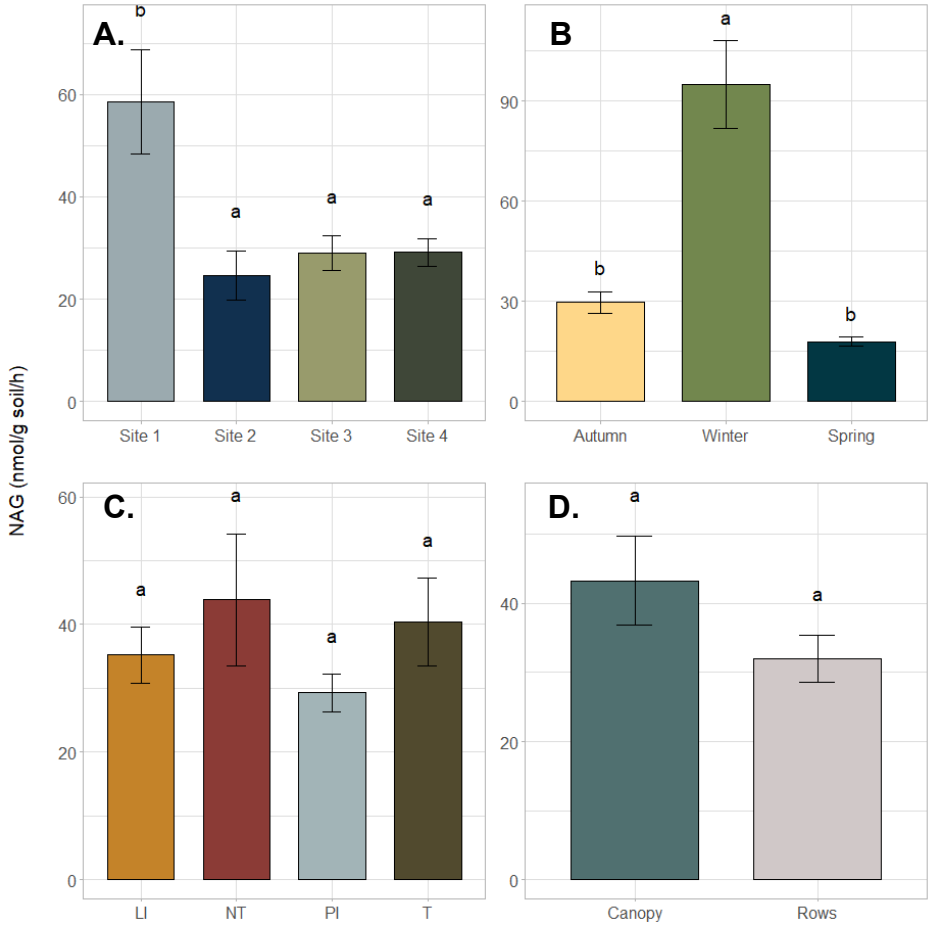

Figure 44: Activity of NAG based on four factors: A. study region, B. season, C. treatment, and D. location—represented in four separate plots. Error bars represent the standard error. Different lowercase letters represent significant statistical differences based on Tukey's test (p<0.05).

Table 18: One-way ANOVA results for NAG activity for each of the studied factors.

Factor	F-value	p-value
Site	5.63	0.001 **
Season	49.19	<0.001 ***
Treatment	0.81	0.49
Location	2.44	0.12

Significance codes: “ . ” <0.1, “ * ” <0.05, “ ** “ <0.01, “ *** “ <0.001

Table 19: Pairwise comparison results for NAG activity based on the one-way ANOVA, using Tukey HSD test (p<0.05).

Factor	Diff	p adj.
	Site	
Site 2 – Site 1	-33.96	0.006
Site 3 – Site 1	-29.65	0.027
Site 4 – Site 1	-29.50	0.005
Site 3 – Site 2	4.31	0.982
Site 4 – Site 2	4.47	0.971
Site 4 – Site 3	0.16	0.999
	Season	
Winter - Autumn	65.25	<0.001
Spring - Autumn	-11.73	0.178
Spring - Winter	-76.98	<0.001
	Treatment	
NT – LI	8.70	0.891
PI – LI	-5.90	0.961
T – LI	5.18	0.970
PI – NT	-14.61	0.468
T – NT	-3.52	0.983
T – PI	11.09	0.639
	Location	
Rows - Canopy	-11.29	0.12

NT = no-till; LI = no-till with pruning’s incorporation and legumes intercropping; PI = no-till with pruning’s incorporation; T = mechanical plough.

To delve further into the factors influencing NAG activity, the effects of combined factors was tested (Table 20). As demonstrated in Figure 45, same seasonal trend was observed across all sites, with NAG activity at Site 1 during winter (137.51 nmol/h/g) standing out and is significantly higher than in seasons across sites. Similar seasonal

pattern was evident in the interactions between season and management practices (Fig. 46). According to the results, the highest overall NAG activity occurred under the conservation treatment NT during winter (143.83 nmol/h/g), while the lowest occurred under the same treatment during spring (11.17 nmol/h/g). During autumn, highest activity was documented under NT treatment (35.84 nmol/h/g) and lowest under the conventional treatment (19.17 nmol/h/g). In both winter and spring, the highest and lowest activities occurred under conservation treatments

Table 20: NAG activity multi-way ANOVA results for interactions of the studied factors.

Interactions	F-value	p-value
Site – Season	19.03	<0.001 ***
Site - Treatment	2.60	0.014 *
Season - Treatment	13.65	<0.001 ***
Site – Season – Treatment	8.84	<0.001 ***
Site – Season – Treatment - Location	6.20	<0.001 ***

Significance codes: “ . ” <0.1, “ * ” <0.05, “ ** “ <0.01, “ *** “ <0.001

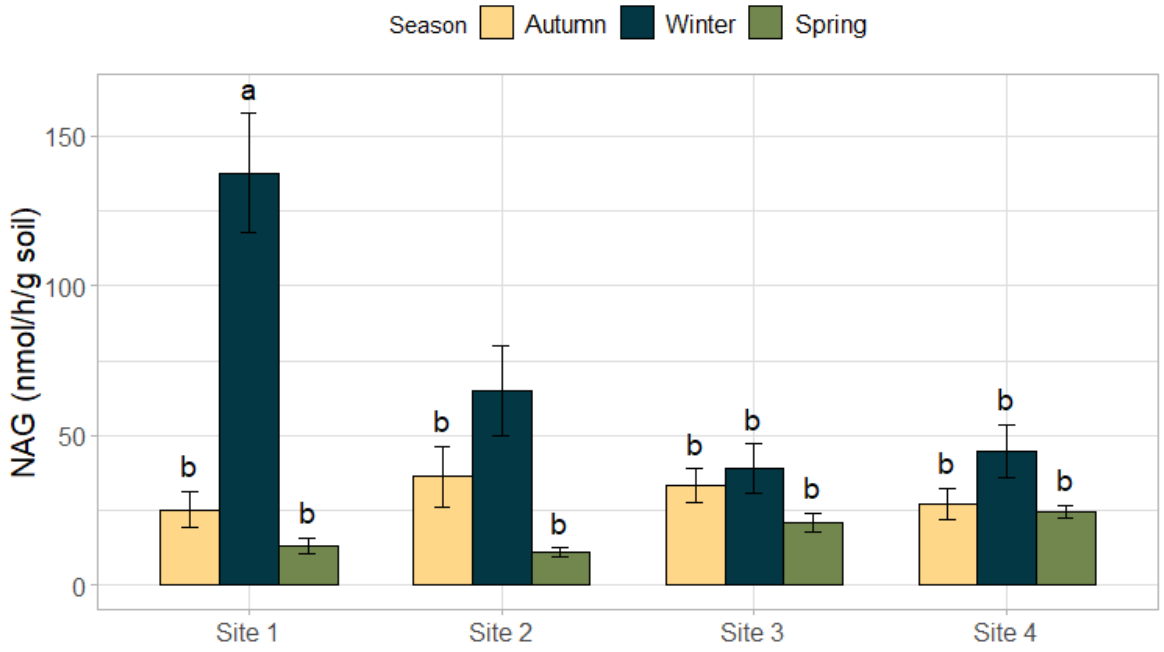

Figure 45: Average NAG activity of the experimental sites during autumn (yellow), winter (blue) and spring (green) sampling. Error bars represent the standard error for the combined effect of Site and Season. Different lower-case letters on top of bars indicate significant difference for the specific interaction of Site and Season, based on Tukey post hoc test (p<0.001).

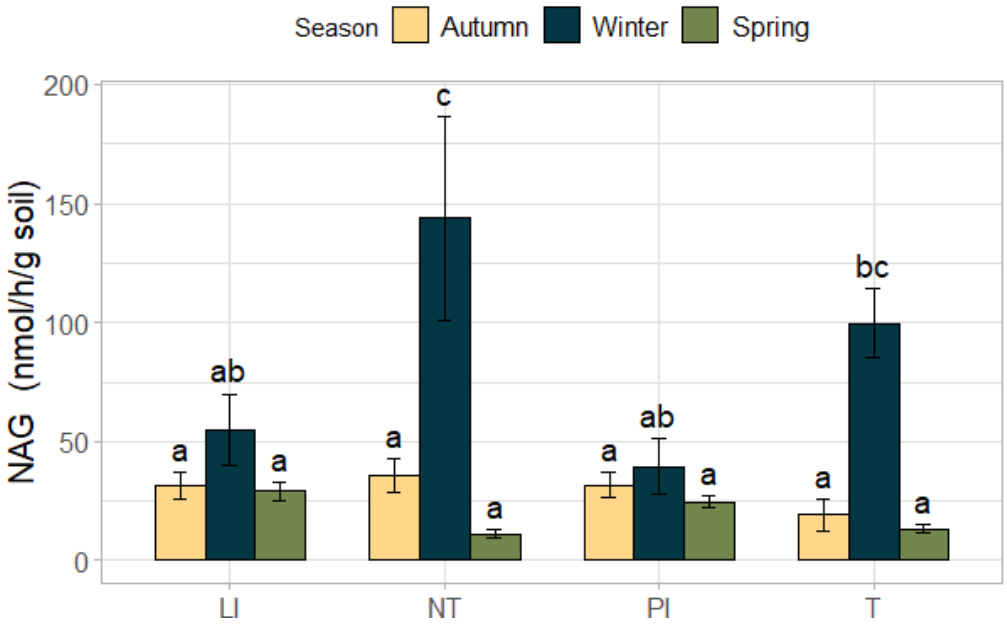

Figure 46: Average NAG activity under different agronomic management practices during autumn (yellow), winter (blue) and spring (green) sampling. Error bars represent the standard error for the combined effect of Site and Season. Different lower-case letters on top of bars indicate significant difference for the specific interaction of Site and Season, based on Tukey post hoc test ($p<0.001$).

When examining the seasonality of management practices at each specific experimental site, we observe that the trend shifts slightly (Fig. 47). In Site 1, the highest NAG activity occurred during winter for both treatments (143.83 nmol/h/g under NT and 132.91 nmol/h/g under T). In the conservation treatment, the lowest activity was detected during spring (12.07 nmol/h/g), whereas for the conventional treatment occurred during autumn (11.68 nmol/h/g). In Site 2, both NT and T treatment followed the same pattern, with higher activity in autumn (41.29 nmol/h/g and 31.27 nmol/h/g, respectively), and lower in spring (11.75 nmol/h/g and 10.30 nmol/h/g). A similar trend was observed in Site 3, with the highest NAG activity during winter (39.05 nmol/h/g) and lowest during spring (20.84 nmol/h/g). Although NAG activity in Site 4 also peaked during winter, under both PI and T treatments, spring activity (29.21 nmol/h/g and 13.98 nmol/h/g, respectively) is slightly higher than in autumn (27.39 nmol/h/g and 12.53 nmol/h/g). The LI treatment of Site 4 follows the same trend as Site 3.

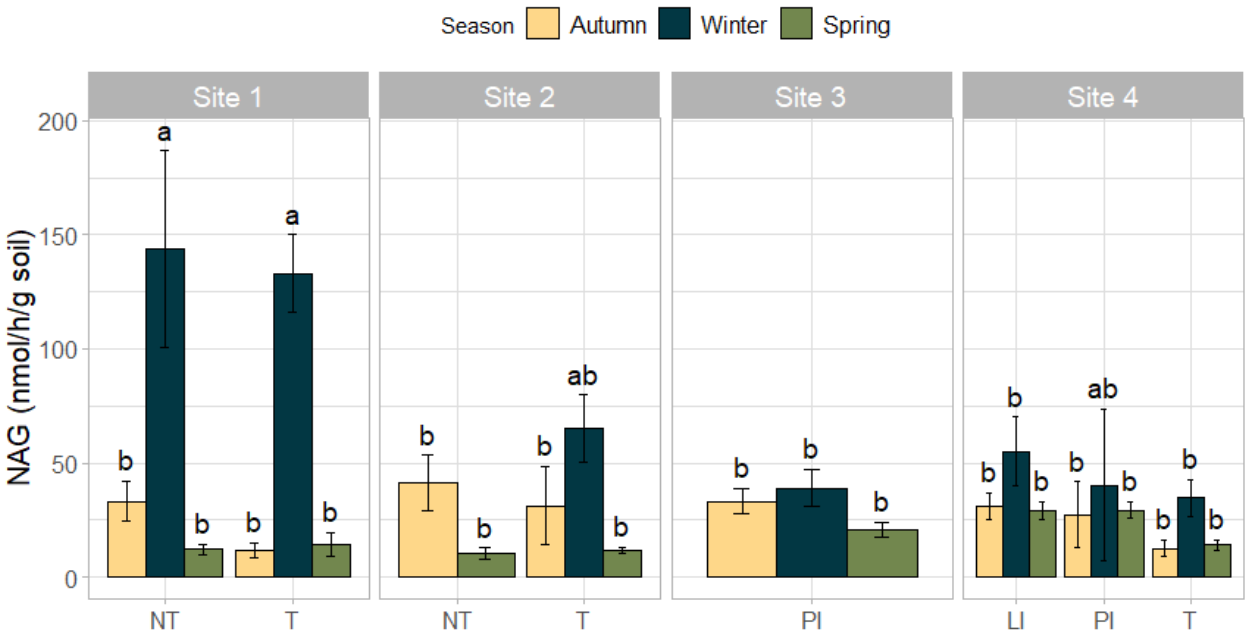

Figure 47: Average NAG activity of the experimental sites for autumn (yellow), winter (blue) and spring (green). Error bars represent the standard error for the interactions of Site, Season and Treatment. Different lower-case letters on top of bars indicate significant difference based on Tukey post hoc test ($p<0.05$).

3.5.5 Soil extracellular enzyme activity – synopsis

Four hydrolytic enzymatic activities were measured and analysed to assess the conversion of olive orchards from conventional to conservation tillage. Additionally, the response of the selected enzymes to seasonality was monitored and assessed for three consecutive seasons in four selected experimental fields, sampled between rows and below olive tree canopies.

The activity of phosphatase was significantly higher than the rest of the enzymes (Fig. 48), implying increased reaction catalysis without diminishing the importance of the rest of enzyme activities and their specific biochemical functions. The reduced activities of NAG and XYL indicate decreased OM decomposition and nutrient cycling in the soil, attributed to low OM availability and soil water stress.

Results revealed that study region and seasonality had the highest effect on enzymatic activity overall, both coupled and as individual factors (Fig. 48). The general pattern of enzyme activity concerning seasonality shows an increase from autumn to winter sampling and a decrease from winter to spring. The effect of agronomic management practices slightly favoured no-tillage, but the resulting activities were not statistically significant. When the combined influence of study region, seasonality and management on enzyme activities was explored, no distinct patterns were observed.

For the most part, conventional treatments had slightly better effect on enzyme activities, with some site-dependent exceptions (Fig. 49). For example, phosphatase activity decreased during winter in Site 3 under conventional treatment, while in Site 4 under conventional treatment reached highest during spring.

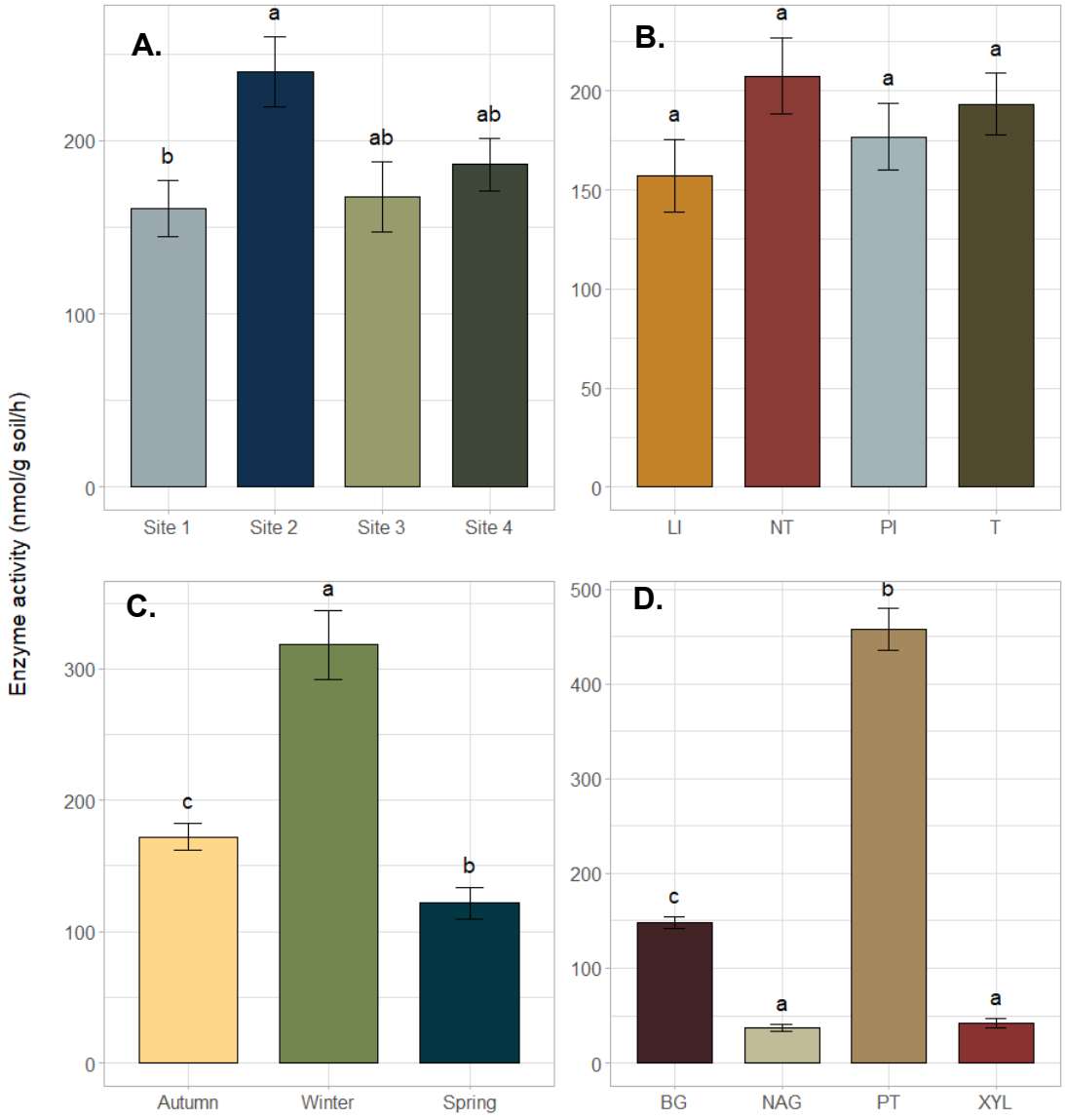

Figure 48: Average enzyme activity across: A. studied regions, B. agronomic management practices, C. seasons. D. Comparison between the overall activities of measured enzymes. Error bars represent standard error. Different lower letters indicate statistical significance (Tukey HSD, $p<0.05$).

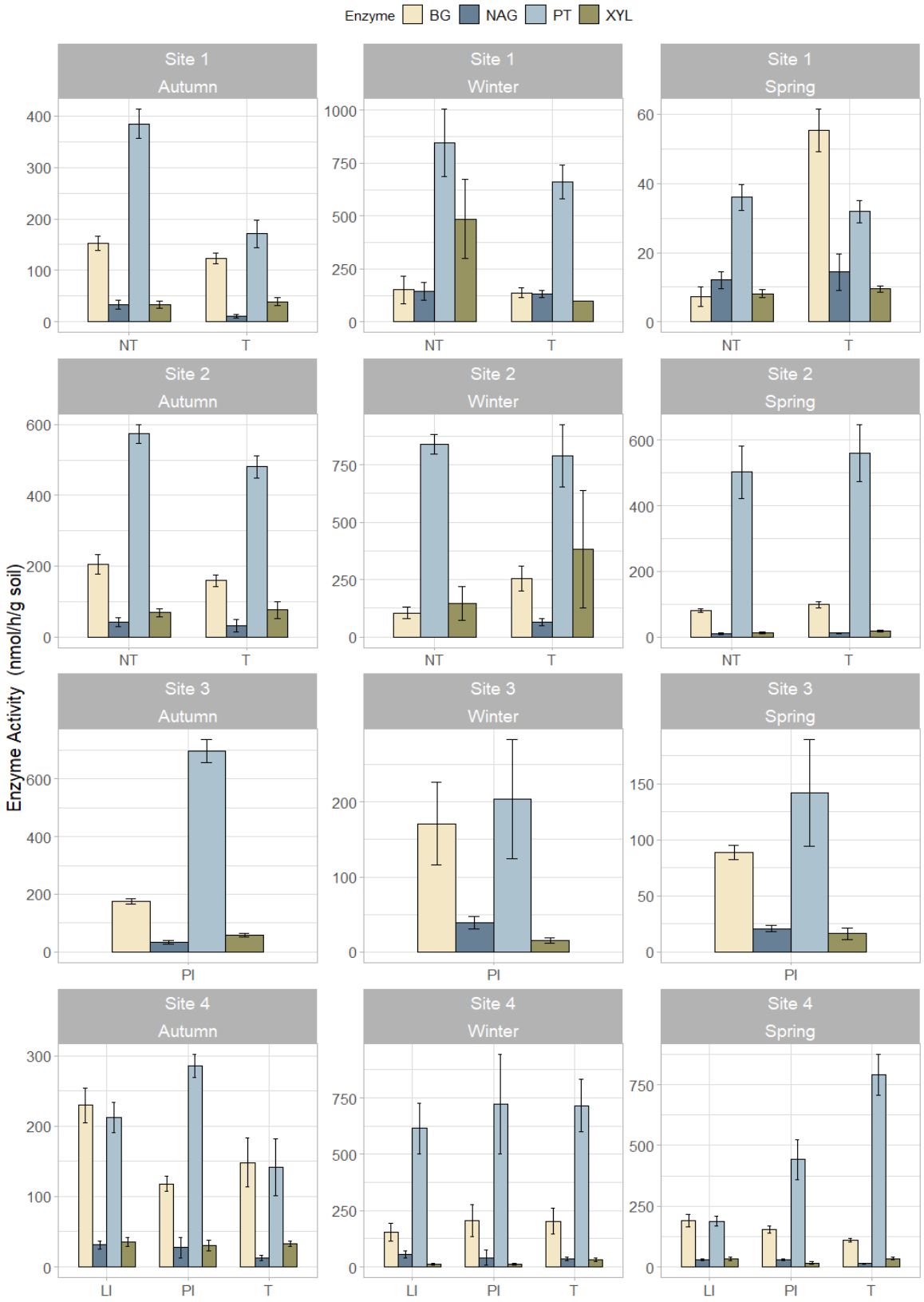

Figure 49: Enzyme activities influenced by study region (rows), seasonality (columns) and agronomic management practices. Different bar colours indicate BG (yellow), NAG (dark blue), PT (light blue) and XYL (green) activities. Error bars represent standard error.

4. Discussion

4.1 Soil carbon stocks

Soil organic C has central role in a range of soil functions, such as microbial activity and nutrient cycling, and is therefore pivotal for food security (Stockmann et al., 2015). Anthropogenic influence on agroecosystems, coupled with biotic properties and abiotic variables, create varied site-specific circumstances, making general assumptions for distribution and determinant influences on SOC challenging (Luo et al., 2017; Jendoubi et al., 2019). In Mediterranean agroecosystems, soils often exhibit decreased SOC contents due to limited primary productivity and conditions that favour rapid microbial decomposition of organic matter. Converting agroecosystems from conventional to conservation management practices has the potential to enhance soil aggregation processes, reduce degradation, and therefore increase SOC content (Abbas et al., 2020).

Similar to the findings of Álvaro-Fuentes et al. (2008), the adaption of conservation practices increased surface SOC content, except at experimental Site 2, likely due to crop residue accumulation and lack of soil disturbances. Likewise, Abbas et al. (2020) reported that in their study, SOC increased in the upper 15 cm layer under no-till with mulch addition. Site-specific results of SOC content enhancement, especially in short period of time since the shift from conventional to conservation (Site 1, ~2 years), highlight the potential improvement in soil fertility which may be achieved through pruning's incorporation (Repullo et al., 2012) and legumes intercropping (Wang et al., 2025).

When the examined study region, agronomic management practices and seasonality were all taken into consideration, a similar pattern could be observed, besides the conservation treatments of Site 2 and Site 3. Although climate is among the primary factors influencing plant growth and consequently affecting the potential C input to soil and microbial decomposition processes, its impact is further shaped by agricultural practices such as residue management and tillage (Luo et al., 2017). Furthermore, based on SOC content, Site 1 and Site 4 responded positively to the land management change from conventional to conservation, demonstrating SOC enhancement and positive outcomes from soil disturbances absence (Vázquez et al., 2020), organic material addition (Michalopoulos et al., 2020), and legumes intercropping. In a study

published by Hu et al. (2023), legumes cover crops has a significant increase in SOC compared to no cover crops (23%), which they explained based on changes in composition of microbial necromass C.

The higher SIC content observed in experimental Sites 1, 2 and 4 is consistent with scientific literature for arid and semi-arid regions, especially in the Mediterranean basin (Apesteguia et al., 2018; Leogrande et al., 2021). A meta-analysis conducted by Niu et al. (2023) highlights the influence of agronomic practices on IC, where NT and plastic mulching film increased the SIC content in the plough soil layer of dryland fields. This trend may be reflected in experimental sites 2 and Site 4, where higher SIC was observed under conservation tillage, though due to the lack of a consistent pattern in the across the studied fields, no definitive conclusion can be drawn.

4.2 Microbial biomass

Soil ecosystem is heavily influenced by microclimatic factors and anthropogenic activities such as land use and management practices. Due to their sensitivity and rapid response to agronomic management change, biological indices are often utilised as early indicators. In the present study, soil microbial biomass C served as an early indicator of changes in f soil biological activity in respect to study region, seasonality, management practice and location. When the abovementioned factors were examined in vacuum, they were found to significantly influence MBC, besides the sampling location, which showed no statistical significance between the samples taken below canopy and those between tree rows.

The complexity of soil matrix and its microclimatic conditions result in unpredictable responses of SMB. A study by Evangelou et al. (2020), conducted in Lesbos Greece, highlighted the significant effect of land use history coupled with seasonality on SMB in Mediterranean agroecosystems, focusing on conventional long-term management (>40 years). Specifically, they documented highest SMB during autumn and lowest during summer. In our case, two seasonal patterns of MBC emerged, a gradual increase with each passing season and higher MBC content during winter.

The positive impact of the management practices shift from conventional to conservation on SMB was evident, especially in Site 4 where organic matter was

returned to no-till soils in the form of pruning's incorporation and legumes intercropping, creating favourable conditions for the microfauna (Fig. 22) (Zuber & Villamil, 2016). Furthermore, MBC was found positively correlated to SOC for both autumn and winter, as it was expected (Fig. 23). This correlation could explain the higher results of SMBC under conventional treatment in Site 2. Li Y. et al. (2018) attribute such increase of MBC under conservation treatment to more favourable environment for fungal hyphae development and overall abundance, due to less soil disturbances and surface residue retention. Their meta-analysis highlights that conservation practices are a promising strategy for MBC increase, with varying magnitudes depending on the environmental factors (Li Y. et al., 2018).

4.3 Soil extracellular enzyme activity

Enzyme assays provide a snapshot of soils' potential to catalyse specific reactions though previously produces and stabilized enzymes or directly living cells present at the time of sampling (Nannipieri et al., 2018). Even though their sensitivity determines them as significant indicators of soil health, due to their substrate-specific responses, it is required to monitor different enzymes to draw a conclusion (Zuccarini et al., 2023). To gain insight on the short-term effect of tillage shift from conventional to conservation management practices and their seasonal variability, four hydrolytic enzyme activities were monitored at four experimental sites and two sampling locations. Due to the complexity of soil matrix and its site-specific environmental conditions, no consistent pattern could be observed across all enzyme activities and factors. Reduced soil moisture content may contribute to lower activity, as water scarcity limits microbial activity by reduced substrate mobility, a major constrained for hydrolytic enzymes (Gomez et al., 2020; Zuccarini et al., 2022).

When the EEAs were analysed without considering the interactions among factors, seasonality emerged as the only variable that significantly influenced all enzymatic activities, whilst sampling location had no significant effect. Zuccarini et al. (2019) highlight the role of seasonality as the driver of microbial composition, nutrient demand and overall activity shift. The activities of BG and XYL were additionally influenced by treatment, supporting previous findings as indicators of management effect on soil ecosystem (Scott et al., 2010). As discussed in Chapter 4.5.5, results slightly favour

NT over conventional tillage, although differences were not statistical significance (Fig. 47).

In the case of BG activity, no clear positive response was observed with the shift from conventional to conservation across seasons and experimental sites (Fig. 30). The weak or absent correlation of BG and SOC on a regional scale could be attributed to different factors, such as the quality and availability of substrate, the microbial community structure, and local environmental conditions (Cenini et al., 2016). Resulting XYL activity exhibited two contrasting patterns, with highest activities in c. Crete and lowest in w. Crete during winter (Fig. 37). The general seasonal pattern of PH activity under conventional and conservation treatment peaked during winter but reached lowest during spring (Figure 40). Through an experiment conducted in a Mediterranean shrubland, Sardans et al. (2007) highlight the influence of decreased moisture content coupled with low temperatures on root-surface PH activity, suggesting an upcoming decrease in P-uptake rate from plants, due to drier conditions. Similar seasonal pattern to PH was detected for NAG activity with high variability during winter (Fig. 45).

5. Conclusions

Concluding this study, it is important to highlight the varying responses of soil organic C, soil microbial biomass and extracellular enzyme activity to the short-term transition from conventional to conservation management practices in Mediterranean agroecosystems. Several consistent patterns emerged despite the strong influence of site-specific and climatic variables.

Consistently with scientific literature, conservation practices, especially those with added organic matter in the form of pruning incorporation or legume intercropping, led to higher soil organic C due to the reduced disturbances and improved soil structure. While soil inorganic C was reported higher in most sites as it is common in Mediterranean, it was not linked back to applied management practices.

Soil microbial biomass C emerged as a sensitive measure of change in soil agroecosystems. Generally, MBC was greater under conservation practices, with distinct seasonal patterns. The positive correlation between MBC and SOC underlines the interlinked relation of soil C and microfauna.

The results of enzyme activity highlight their strong seasonal variability. Whilst β-glucosidase and β-xylosidase responded to agronomic management change, no clear pattern was drawn. Furthermore, it underlines the need for multiple enzyme assays to assess the bigger picture.

While the differences between regions and seasons emphasize the magnitude of local conditions, the findings suggest that conservation practices and particularly those incorporating organic matter inputs, benefit the soil C dynamic and biological activity, even within a short transition period.

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