EFFECTS OF ARBUSCULAR MYCORRHIZAL FUNGI INOCULATION AND COVER CROP ON SOIL CARBON DYNAMICS AND MICROBIAL COMMUNITIES IN A MEDITERRANEAN LEMON ORCHARD

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Anna Rodriguez-Paiatsyka

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AUTHOR: Anna Rodriguez-Paiatsyka

DATE SUBMITTED: December 2023

PROJECT ADVISOR Charlotte Decock, Ph.D.,
Associate Professor of Soil Health and Fertility
ABSTRACT

Effects of arbuscular mycorrhizal fungi inoculation and cover crop on soil carbon dynamics and microbial communities in a Mediterranean lemon orchard.

Anna Rodriguez-Paiatsyka

Plant-associated fungi such as arbuscular mycorrhizal fungi (AMF) have the potential to sequester carbon (C), improve soil aggregation, and promote plant health. Due to multiple benefits of AMF to plant and soil health, AMF has gained much attention leading to a rapidly expanding market in mycorrhizal bio-stimulants intended to improve crop yield, root development, and soil health in horticultural crops, including citrus. However, there is limited information on how to inoculate a mature citrus orchard, and how inoculation of a mature orchard with AMF affects C sequestration. In this study, we planted a cover crop inoculated with AMF in a mature lemon orchard and investigated the influence of the cover crop and AMF inoculation on C dynamics and the soil microbial community across the orchard floor. The experimental design was a Randomized Complete Block Design (RCBD) with three blocks and the following treatments applied in the alleyways: a cereal cover crop (Secale x Triticum L.) inoculated with AMF (Rhizophagus intraradices) at seeding, a cereal cover crop without inoculation, and a control where orchard alleys were left fallow. After two years of treatment implementation, soils were collected from each treatment plot from two functional locations, the tree row and the alley way, and two depth increments of 0-6 and 6-18 inches in order to assess soil C dynamics. To assess legacy effects of three years of practice implementation on soil C dynamics and microbial community structure, soils were collected at 0-6 inches depth from four functional locations: location one was between two trees on a berm, location two was in the transition section where the berm ends but no cover crop is grown, location three was on the edge of the cover cropped area (weeds in control plots), and location four was in the center of the alley row. AMF inoculation had no significant effects on soil C dynamics indicators such as total soil C, permanganate oxidizable carbon (POXC), mineralizable carbon (Min C), and soil aggregation. Likewise, AMF inoculation had no significant effect on microbial community biomass assessed with soil phospholipid fatty acids (PLFA) and neutral lipid fatty acids (NLFA). We attributed similarities between treatments to the non-inoculated cereal cover crop and weeds in the control treatment promoting native AMF. Across treatments, alley rows supported higher microbial community biomass and had higher total soil C (%), POXC (mg C/kg soil), and Min C (mg C/kg soil/day) than the tree row after three years of treatment implementation. After two years of treatment implementation, total soil C, Min C, and small macroaggregates (%) were higher in the topsoil in the cover cropped alley row compared to the topsoil in the three rows. We attribute the greater values for C cycling indicators and microbial abundance in the alley rows compared to the tree rows to positive impacts of tree prunings, weeds and cover crops and negative impacts of dry wet cycles in the alley versus tree rows, respectively. Correlation analysis showed that AMF and saprophytic fungi were significantly correlated with bacteria but not among themselves suggesting potential fungal-bacterial cooperation and niche differentiation between saprophytic fungi and AMF. In addition, correlation analysis showed that AMF and saprophytic fungi NLFA biomass was not
significantly correlated with soil C indicators which contrasts other findings where fungi were key organisms in soil C accumulation. To summarize, AMF inoculation had no significant effect on soil C dynamics or the microbial community whereas differences in management between the tree and alley rows had a strong influence on soil C dynamics, microbial community biomass, and native AMF promotion in the lemon orchard.

Keywords: AMF, inoculation, soil carbon, cover crop, POXC, soil aggregation, soil health, microbial community, PLFA, NLFA
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CHAPTER 1: INTRODUCTION

Citrus crops play an important role in global fruit production, and are grown in many regions in the world with various climates including subtropical, tropical, and Mediterranean (FAO, 2021). These crops are often intensively managed leading to soil health degradation and loss of soil carbon (C) in the form of carbon dioxide (CO₂) (Niu et al., 2021). Soils play an important role in the regulation of greenhouse gas emissions as they have the potential to store CO₂ that was captured by plants from the atmosphere and assimilated into plant tissues in an organic form; however, whether soil organic carbon (SOC) is increased or depleted depends on the choice of soil management (Lal, 2004; Lal et al., 2015; Weil & Brady, 2009). In citrus growing regions of the USA, growers are encouraged to incorporate management practices that increase SOC and improve soil health (Haynes, 2022; Teater, 2022). Assessing the potential for C sequestration and improvement of soil health in citrus orchards is a vital component of sustainable citrus production and of mitigation of climate change.

Plant-associated fungi such as arbuscular mycorrhizal fungi (AMF) have the potential to sequester C, improve soil aggregation, and promote plant health (Chen et al., 2018; Parihar et al., 2020; van der Heijden et al., 2015). About 74% of all plant species can form associations with AMF from the Glomeromycota clade (Brundrett, 2009; Smith & Read, 2008). After contact and root penetration, AMF form symbiotic associations with a host plant inside the plant’s root cortical cells by forming a highly branched tree-like structure called arbuscules where nutrients are transferred to plants from AMF and C from plants to the AMF (Chen et al., 2018; Smith & Read, 2008; van der Heijden et al., 2015). AMF produces highly branched thread-like mycelia which spreads through soil
creating extensive mycelia networks; thus, AMF association allows plants to acquire
nutrients and water outside of the root depletion zone (Smith & Read, 2008). Beside
plant nutrition, AMF, can provide numerous potential benefits to plants and soil structure
such as root/plant protection from soil-borne pathogens, reduced soil erosion, improved
soil aggregation, reduced nutrient leaching and increased yield (Chen et al., 2018). Citrus
trees create associations with AMF (Wu & Srivastava, 2017; Xi et al., 2022) which has
been shown to improve crop nutrition (Wu & Zou, 2009), enhance tolerance against
abiotic stress such as drought (Wu et al., 2019), and induce better root development (Wu
et al., 2012).

The AMF and plant symbiosis results in a direct flow of C from the atmosphere to
the soil in the form of photosynthates, with plants allocating an estimated 4-25% of their
photosynthates to AMF (Hobbie, 2006). AMF contribute to the soil C pool by producing
C-rich extra-radical (mycelia) and intra-radical biomass and mycelia exudates, and by
improving soil aggregation that in turn protects soil organic matter (SOM) from
decomposition (Rillig, 2004a; Zhu & Miller, 2003). AMF play an important role in soil
macroaggregate formation by the ‘sticky-string-bag’ mechanism (Miller & Jastrow,
2000), where mycelia with their filamentous nature and ability to grow between small
spaces, entangle and enmesh soil particles and smaller soil aggregates. In addition,
mycelia exudates act as binding material in aggregate formation. Soil macroaggregates
protect organic debris from decomposition leading to reduced C loss (Miller & Jastrow,
2000; Rillig, 2004b; Zhu & Miller, 2003). A five week- laboratory incubation showed
that AMF had a stabilizing effect on large macroaggregates (Morris et al., 2019) and field
experiments showed positive correlations between large macroaggregates and AMF
presence in the soil (Lin et al., 2023; Wilson et al., 2009). Several studies have proposed that AMF produce a C rich glycoprotein-like substance called glomalin. Glomalin content in soil is often measured through different methods including glomalin related soil proteins (GRSP), total glomalin related soil proteins (T-GRSP), Bradford-reactive soil protein (BRSP), and immunoreactive soil protein (IRSP) (Irving et al., 2021). Even though glomalin has been widely studied, its nature and relation to AMF is still not well understood (Holátko et al., 2021; Irving et al., 2021; Rillig, 2004a; Wright & Upadhyaya, 1998). Despite uncertainties about the nature of glomalin, many studies have found a positive influence of glomalin on soil aggregation and other benefits to soil health (Holátko et al., 2021; Hossain, 2021). In addition, glomalin is often considered to be a major contributor to the SOM pool (Pei et al., 2020; Q. Wang et al., 2020; Y.-J. Wang et al., 2023; Wright & Nichols, 2002).

Over the past decade, our understanding of SOM dynamics has shifted from the paradigm where resistance of plant residues decomposition was thought to be the dominant driver of C sequestration to a new consensus recognizing the importance of dead microbial biomass (necromass) in SOC stabilization (Khan et al., 2016; Miltner et al., 2012; H. Wu et al., 2023). The soil microbial pump (MCP) was coined as a concept where C from plant residues is first transformed into microbial biomass during plant decomposition by soil organisms, after which it is stabilized in soil as microbial necromass (Liang, 2020). The contribution of AMF to soil C dynamics is different than saprotrophic soil organisms due to a direct flow of C from the plant host to the AMF biomass. Earlier studies focusing on AMF mycelia determined that hyphal biomass contributes 20-30% to total soil microbial biomass (Leake et al., 2004; Olsson et al.,
Even though AMF mycelia attached to plant roots were found to have a short residence time of 5 to 6 days (Staddon et al., 2003), it is believed that recalcitrant carbohydrate-based chitin as part of mycelia remains after mycelia decomposition and contributes to more stable soil C pools due to its longer residence time in soil (Gleixner et al., 2002; Morton, 2021; Taylor & Sinsabaugh, 2015).

Saprophytic soil microorganisms, fungi and bacteria, participate in breaking down dead plant materials into simple molecules that can be used by soil microorganisms for biomass production. Beside dead plant residue, microbial residues and root rhizodeposition can be used by soil microorganisms as building blocks for microbial biomass (Fuhrmann & Zuberer, 2021; Hemkemeyer et al., 2021). In contrast to saprophytic soil microorganisms, AMF receive C from the host plants which allows AMF mycelia to extend far from the host exploring bulk soil (Smith & Read, 2008; Wipf et al., 2019). The difference in nutritional needs allows soil microorganisms to occupy various soil niches. For example, plant roots deposit C-rich rhizodeposits into the soil leading to the creation of a distinct environment compared to bulk soil where major resources for microbial use are litter-derived older materials (Weil & Brady, 2009; Yuan et al., 2021). In orchards with bare soil around the trees, relative abundance of soil microorganisms can therefore be reduced compared to the cover cropped alleys due to absence of carbon input from the cover crop (Rodriguez-Ramos et al., 2022).

Since soil microorganisms share soil niches, they may compete or cooperate for resources and space (Tecon & Or, 2017). There is evidence that AMF can influence other microbial groups (Fall et al., 2022) such as decomposers (Chowdhury et al., 2022) or shift a community structure by suppressing fungi and promoting bacteria (Monokrousos et
al., 2020). In a microcosm study with nutrient poor soil, AMF and bacterial coinoculation showed higher litter decompostion than AMF alone or AMF and saprotrophic fungi indicating a potential cooperation between AMF and bacteria (Cao et al., 2022). In a greenhouse study using wild oat grass and soils collected from California annual grassland where the domenant type of vegetation was *Avena* species, AMF-bacterial coocurrence was observed at a higher rate in bulk compared to rhizosphere soil, further supporting a potential cooperation between AMF and bacteria (Yuan et al., 2021).

Research on the influence of AMF on soil C dynamics has predominantly focused on studying native AMF in various systems under different management types (Agnihotri et al., 2021; Huang et al., 2023; Lin et al., 2023; P. Wang et al., 2016b; Wilson et al., 2009; Y. Zhang et al., 2022). In agricultural systems, abundance of AMF can be negatively impacted by intensive production leading to a decrease of AMF spores and infective mycelium; thus, native AMF is often promoted by manipulation of field management or by inoculation when AMF abundance or diversity is limited. (Bowles et al., 2016; Verbruggen et al., 2013). Due to multiple benefits of AMF to soil and plant health, AMF has gained much attention leading to a rapidly expanding market in mycorrhizal bio-stimulants focusing on improvement of crop yield and root development of horticultural crops (Chen et al., 2018; Igiehon & Babalola, 2017). In case of trees, the most common approach to study AMF effect on trees, including citrus, is by inoculating seedlings in pots or during transplantation into the field (Wang et al., 2016; Wu & Zou, 2009), since reaching roots of mature trees can be challenging and often requires drilling holes around each plant to reach the root zone (Mamatha et al., 2002) or specialized equipment such as a soil injector (Appleton et al., 2003). In a mature orchard, a potential
strategy to establish AMF from inoculum is through inoculation of the cover crop (Benitez et al., 2016; Chen et al., 2018) as AMF is able to colonize different plant species (low host specificity) and establish mycorrhizal networks across long distances by connecting multiple plants (Smith & Read, 2008; van der Heijden et al., 2015; Wipf et al., 2019). In a chamber experiment, white clover, as a host plant inoculated with AMF (Diversispora spurca), established AMF connection with non-inoculated trifoliate orange seedlings (Z.-Z. Zhang et al., 2015) demonstrating the potential of colonization of citrus trees roots with AMF through an inoculated cover crop. In addition, various cover crops can successfully promote native AMF abundance and crop or tree root colonization in annual and perennial agricultural systems (Bowles, Jackson, et al., 2016; Buyer et al., 2010; Finney et al., 2017; Martínez-García et al., 2018; Rankoth et al., 2019; Rodríguez-Ramos et al., 2022; Thapa et al., 2021; Trinchera et al., 2021; Turrini et al., 2017; Vasilikiotis et al., 2020; P. Wang et al., 2011, 2016b; H. Zhang et al., 2023). However, research on effects of AMF in perennial cropping systems remains limited (Wang et al., 2016; Xi et al., 2022). Moreover, many studies that focus on the effect of AMF inoculation on soil C dynamics were performed in the laboratory or greenhouse settings (Fang et al., 2020; Hu et al., 2014; Jeewani et al., 2021; Morris et al., 2019; Subramanian et al., 2019; Wang et al., 2023) and the role of a cover crop on promoting native AMF compared to the potential of cover crops to facilitate AMF inoculation in a mature orchard is poorly understood.

In this study, we aim to address knowledge gaps about the potential of cover crops and inoculation with AMF to sequester C in a Mediterranean lemon orchard. The specific objectives of this research were 1) to investigate the effects of cover cropping and
inoculation with arbuscular mycorrhizal fungi on soil C dynamics, 2) assess legacy effects of three years of practice implementations on microbial community structure, and 3) to investigate the relationship between microbial community structure and C sequestration across the orchard floor. To this end, a three-year field trial was established with the following treatments: a cereal cover crop inoculated with AMF at seeding, a cereal cover crop without inoculation, and a control where orchard alleys were left fallow. In the first two years, we distinguished two functional locations within each treatment plot; the tree row where soil on the berms was kept bare as a standard practice and the center of the alley row where treatments were applied. In the third year, we added two more functional locations to expand coverage of the orchard floor, the area next to the berm that appeared compacted and was clear of any vegetation, and the edge of the cover cropped area where weed pressure was typically high. To assess C pool dynamics, we measured total soil C, permanganate oxidizable carbon (POXC) and mineralizable carbon (Min C). While total soil C can track C sequestration over time, its turn over time is slow. In contrast, POXC and Min C are C cycling indicators that are more sensitive to short-term management-induced changes and that can be linked to the role of microorganisms in C dynamics (Hurisso et al., 2016). POXC measures the fraction of the active C pool that may be predominately of microbial origin and is likely to be stabilized in soil, while Min C measures the fraction of the active C pool that tends to be mineralized by soil organisms (Hurisso et al., 2016). Soil aggregation is used as another C sequestration indicator because microbial and plant based C rich binding materials are protected within aggregates leading to soil organic C stabilization (Morris et al., 2019; Six et al., 2000). Soil microbial biomass was assessed by measuring phospholipid fatty
acids (PLFAs) and neutral lipid fatty acids (NLFAs) where PLFAs are components of cell membranes and found in bacteria and fungi and NLFAs are associated with storage of fungi only (Bååth, 2003). The unique molecular structure of PLFAs and NLFAs follows the phylogeny of soil organisms and allows researchers to estimate the live biomass of different microbial groups and evaluate community structure on a broad scale (Lekberg et al., 2022; Nkongolo & Narendrula-Kotha, 2020). Various biomarkers were established to identify specific microbial groups, but some biomarkers can be found in two different groups leading to possible misinterpretation of data. Biomarker PLFA 16:1 ω5 is used to identify AMF biomass; however, the fatty acid 16:1 ω5 is also found in gram-negative bacteria. In order to minimize misinterpretation of the data, it is recommended to measure NLFA 16:1 ω5 in addition to PLFA 16:1 ω5 as bacteria do not use neutral lipid fatty acids for storage (Lekberg et al., 2022).

We hypothesized that the cereal cover crop and inoculation with AMF will increase total soil C compared to fallowed orchard alleys due to increased inputs of organic matter and improved soil aggregation leading to better protection of soil organic matter from degradation. We further hypothesized that AMF, total bacterial, and saprophytes biomass will be higher in treatments with cover crop compared to the fallowed orchard alleys treatment because the cover crop provides microorganisms with carbon sources required for their function. We also expected that the effect of the cover crop on the microbial community would decrease from the center of the alley row to the tree row. Furthermore, we anticipate that inoculation with AMF would promote bacterial biomass. Due to the potential contribution of microbial necromass to the labile C pool measured with POXC, we hypothesized that AMF, total bacterial, and saprophytes
biomass would be positively correlated with POXC across treatments and locations on the orchard floor. We also expected a positive correlation between AMF, total bacterial, and saprophytes biomass and Min C, associated with their activity in the soil. In addition, we hypothesized that inoculation with AMF would yield a lower Min C:POXC ratio compared to other treatments indicating lower CO$_2$ loss and greater stabilization of newly assimilated C.
CHAPTER 2: MATERIALS AND METHODS

2.1 Field site description and experimental design

The study was conducted at a commercial citrus orchard located in Edna Valley, San Luis Obispo County, California (35.219156, -120.588315) between Fall 2019 and Spring 2022. According to the Web Soil Survey, the soil type at the site is a Cropley clay (Cropley series; fine, smectitic, thermic Aridic Haploxererts) (Soil Survey Staff, 2019). The climate of the region is warm Mediterranean which includes hot, dry summers. This type of climate is classified as Csb based on the Köppen-Geiger climate classification (Kottek et al., 2006). The average air temperature and precipitation is based on the nearest CIMIS weather station approximately 7 miles to the northwest from the orchard (#52, San Luis Obispo, California Irrigation Management Information System [CIMIS]).

The mean annual air temperature was 16.94 °C and the mean annual precipitation was 485 mm over the past ten years (2012 to 2022). For the year of 2020, the average maximum and minimum air temperature were 22.8 °C and 9.8 °C, respectively, and the total average precipitation was 281.8 mm. Months that received at least 5 mm of rain were January (10.8 mm), March (146.00 mm), April (64.1 mm), November (14.2 mm), and December (42.8 mm). For the year of 2021, the average maximum and minimum air temperature were 21.6 °C and 9.5 °C, respectively, and the total average precipitation was 545.2 mm. Months that received at least 5 mm of rain were January (183.3 mm), February (7.90 mm), March (30.80 mm), October (54.70 mm), November (8.90 mm), and December (257.20 mm). For the year of 2022, the average maximum and minimum air temperature were 22.6 °C and 9.3 °C, respectively, and the total average precipitation was 379.2 mm. Months that received at least 5 mm of rain were March (18.60 mm), April
(14.00 mm), September (33.00 mm), November (26.00 mm), and December (284.00 mm).

The testing site was a 6-acre block planted with *Citrus limon (L.) Burm. f.* ‘Lisbon Lemon’. The lemon block was planted in 2010, and the drip irrigation system for water and nutrients delivery was installed in 2014 in the tree rows. Nutrients are applied through fertigation once a month for 11 to 12 applications a year, totaling a rate of 200 lb N/acre and 100lb K₂O/acre. During dry summer months, alley rows do not receive any water, and only tree rows receive water and nutrients. The trees are trimmed at the end of summer, and the plant residue is mulched and disked into soil in the alley rows. The block had no history of pre-emergent herbicide application in the alley rows. The trees were planted on berms and berms were kept clear of weeds through herbicide application.

The experimental design was a Randomized Complete Block Design (RCBD) with three blocks and tree treatments in each block resulting in total of 9 plots (Figure 1). Each plot was 0.46 acres and included five alley rows with the middle tree and alley rows being chosen for soil sampling. Soil treatments included a control (C) treatment (bare fallow with no herbicide application), a non-legume cover crop (NLCC) treatment (Triticale cover crop was purchased from Hearne Seed Company in King City, CA; drill-seeded at 110 lbs/acre), and a non-legume cover crop treatment inoculated with arbuscular mycorrhizae fungi (NLCC-M) (Triticale (*Secale x Triticum L.*), 110 lbs/acre inoculated with commercial AMF inoculum of the single AMF species *Rhizophagus intraradices*, 300 propagules/gram) at the rate of 10 lb/acre. The timing of cover crop seeding and AMF inoculation was selected in accordance with winter rain events. Cover crops were seeded in alley rows in early January of 2020 for the 2020 growing season,
late December 2020 for the 2021 growing season, and in early February of 2022 for the 2022 growing season. For inoculated treatments, the AMF inoculum was mixed with the cover crop seed in the seed hopper before planting. In the winter of 2022-2023, early rain promoted germination of cover crop seed that had been produced in previous seasons, and there was no arbuscular mycorrhizae fungi inoculation in the NLCC-M treatment plots. Cover crops were completely rain fed and did not receive supplemental irrigation during dry months. For each cover crop season, the cover crop was terminated by mowing in June.
Figure 1. Experimental design of the study at the commercial orchard in a lemon ‘Lisbon’ block. Blue rectangles represent Control (C) - bare fallow, no pre-emergent herbicide; orange rectangles represent non-legume cereal cover crop (NLCC): Triticale, 110 lbs/acre; green rectangles represent non-legume cereal cover crop + mycorrhizae (NLCC-M): Triticale (*Secale x Triticum* L.), 110 lbs/acre inoculated with single AMF species *Rhizophagus intraradices* (300 propagules/gram) at the rate of 10 lb/acre.

2.2 Soil collection

Baseline soil samples were taken in early January 2020 prior to cover crop planting. Thereafter, soil samples were taken in early January of 2021, mid-January 2022, and mid-February 2023 representing effects following one, two, and three years of cover
crop plantings. In 2020, 2021 and 2022, soil samples were taken with a soil auger equipped with a sliding hammer to a depth of 18 inches (Giddings manual bulk soil core sampler with a diameter of 5 cm; Windsor, CO, USA). Two soil samples were taken in the middle of the alley row (or alley row), and two soil samples were taken between trees in each treatment plot. Each soil sample was immediately split into 0 to 6 inch and 6 to 18 inch depths and placed in labelled polyethylene bags. A total of 36 cores were taken with 18 cores per alley row and 18 cores per tree row yielding 72 samples after the cores were split into the two depths. In this study, findings for the soil samples collected in 2022, representing 2 years following treatment application, are shown.

In February 2023, we modified the sampling scheme to better understand the link between the position on the orchard floor, microbial community structure, and soil C dynamics as affected by the cover crops and AMF inoculation. Composite soil samples were taken from each experimental plot at four functional locations defined as follows: location one was between two trees on a berm, location two was in the transition section where the berm ends but no cover crop is grown, location three was in the cover crop edge (weeds in control plots), and location four was in the center of the alley row (Figure 2). For each composite sample, we collected 15 to 20 cores per functional location per each treatment plot with a 1-inch diameter soil probe (AMS, Inc., American Falls, Idaho, USA) to approximately 6-inch depth.
Figure 2. Modified sampling scheme across the orchard floor. Sampling location one was between two trees on a berm, location two was in the transition section where the berm ends but no cover crop is grown, location three was in the cover crop edge (weeds in control plots), and location four was in the center of the alley row.

2.3 Analyses of soil

Each sample was sieved through an 8 mm sieve (U.S.A. Standard testing sieve; A.S.T.M. E-11 specification, Gilson Company, INC., OH, USA) by hand and left to air dry in a paper bag. Total soil C (%) was determined by first grinding a representative air-dried soil sub-sample in a ceramic mortar and pestle to achieve a homogeneous powder. Next, approximately 1 g of ground soil sample was combusted by using a Vario Max
CNS elemental analyzer (Elementar, Langenselbold, Hesse, Germany) at 900 °C.

Presence of carbonates in the soil samples was tested by applying one to two drops of 1M of HCl acid to a small subsample taken from each soil sample. The test confirmed that inorganic C is present at this site. Soil pH was determined by mixing 10 g of soil and 20 ml of deionized water in a polypropylene 50-ml screw-top centrifuge tube and shaking it for approximately 10 seconds. Next, soil was allowed to settle on the bottom of a centrifuge tube prior to pH measurement. A pH measurement was recorded with a pH Accument AB 150 meter (Thermo Fisher Scientific, Waltham, MA, USA) after 1 minute wait time that allowed the pH meter to stabilize. Electrical conductivity (EC) was determined based on a saturated paste measurement with ECTestr 11+ hand-held meter (Eutech Instruments Pte Ltd., Singapore). Saturated paste EC was converted to EC equivalent in saturated paste extract (dS/m) by using the formula:

\[ EC_{ESP} = 2.7 \times EC_{SP} + 0.8 \]

Permanganate oxidizable carbon (POXC) was determined colorimetrically by using the method described in Culman et al. (2012), adjusted from the method by Weil et al. (2003). Briefly, 2.5 g of air-dried soil was mixed with 18.0 ml of water and 2.0 ml of 0.2M KMnO₄ in a polypropylene 50-ml screw-top centrifuge tube. All samples with method blanks were shaken for 2 min at 240 oscillations per minute and allowed to settle for exactly 10 minutes. Next, 0.5 ml of supernatant was diluted in 49.5 ml of deionized water. The diluted samples (250µL per sample) were placed in a 96-well plate. Four standard stock solutions (0.00005, 0.0001, 0.00015, and 0.0002 M KMnO₄) were used to create a standard curve. Samples and standards were analyzed by an Infinite N Nano+ dual-mode microplate reader at 550 nm wavelength (Tecan Trading, AG, Switzerland).
The concentration of POXC (mg kg\(^{-1}\) soil) was calculated by using the formula in Weil et al. (2003):

\[
POXC = \left[ \frac{0.02 \text{ mol}}{L} - (a + b \times \text{Abs}) \right] \times \frac{9000 \text{ mg C}}{\text{mol}} \times \frac{0.02 \text{ L solution}}{m}
\]

where \(a\) = intercept of the standard curve; \(b\) = slope of the standard curve; \(\text{Abs}\) = absorbance of the sample’s POXC measurement; 9000 = milligrams of carbon oxidized by 1 mole of MnO\(_4\) changing from Mn\(^{7+}\) to Mn\(^{4+}\); and \(m\) = mass of air-dried soil sample in kg.

Mineralizable carbon (Min C), also referred to as soil respiration, was measured by rewetting 10g of air-dried soil to 50% water-holding capacity with deionized water in canning jars with lids equipped with two septa. After a 48-hour incubation period at 20 °C in an IncuMax IC-500R incubator (Amerex Instruments, Inc., Concord, CA, USA), the CO\(_2\) (µmol mole\(^{-1}\)) concentration was measured with a Li-COR Li-850 CO\(_2/)H_2O\) gas analyzer (Lincoln, NE, USA). Four sets of standards were prepared by injecting 0 (blank), 50, 100, 250, 500, 1000, 2500, 5000 µL of high purity (99.999%) CO\(_2\) in the same type of canning jars used for the incubation. Known CO\(_2\) concentrations in the canning jars prepared as standards were calculated based on the volume and concentration of CO\(_2\) injected, the volume of the canning jar, and the ambient concentration of CO\(_2\) in the room. Canning jars prepared as standards were incubated at the same time as soil samples and were used to determine a calibration curve. The CO\(_2\) concentration in the jars was measured with the LI-850 using a closed loop system. Calibration curves accounted for CO\(_2\) in ambient air present in the instrument and closed loop during the measurement. Min C (mg CO\(_2\)-C kg\(^{-1}\) soil day\(^{-1}\)) was calculated based on the difference in CO\(_2\) concentration in the jar after 48h of incubation and ambient CO\(_2\).
concentrations, assuming a linear increase in CO₂ over time. The change in concentration measured in mmol CO₂ mol⁻¹ was converted to mg CO₂-C kg⁻¹ soil day⁻¹ using the ideal gas law, the average volume of the jars (0.241 L), the mass of soil (10g), the incubation temperature (293K), molar masses of C and O, and the incubation time (2 days).

Soil aggregation was determined based on the method described in Six et al. (2000) by manually wet-sieving each soil sample into four aggregate-size fractions: silt and clay (S+C; <53 µm), microaggregate (m; 53-250 µm), small macroaggregate (SM; 250-2000 µm), and large macroaggregate (LM; >2000 µm). Approximately 80 g of air-dried soil was placed in a 2000 µm sieve submerged in the shallow basin with DI water. The sieve was gently lifted from side to side in a rocking motion for 2 minutes. Soil aggregates remained on the sieve were transferred to an aluminum tin labeled as large macroaggregates. Water with other aggregate fractions were transferred into a second basin with the 250 µm sieve with all steps being repeated leading to separation of small macroaggregates. In the last separation step, soil aggregates that remained on the 53 µm sieve were transferred into the tin labeled as microaggregates, and the remaining water in the basin contained the silt and clay fraction. All samples were dried in the oven at 60 °C for several days until fully dry. The weight of each fraction was recorded by subtraction of the tin weight with soil and the empty tin.

2.4 PLFA and NLFA microbial analysis

Analysis of soil phospholipid fatty acids (PLFA) and neutral lipid fatty acids (NLFA) content was conducted by Ward Laboratories, inc. (Kearney, NE). The PLFA procedure is based on the standard method described by Buyer et al. (2010) and adjusted method by Buyer & Sasser (2012). The NLFA procedure follows the same method as for
PLFA except for the lipid separation step, which was adjusted as described in Sharma & Buyer (2015). The first step of PLFA and NLFA extraction included combining 2 g of freeze-dried soil sample and 4 mL of Bligh-Dyer extractant (1:2:0.8 vol:vol:vol) to receive a lipid-containing phase. The next step was to separate lipids into classes by the solid phase extraction (SPE) with a 5:5:1 chloroform:methanol:water fraction for PLFA analysis and a chloroform fraction for NLFA analysis. The resulting phospholipid and neutral lipid fatty acids were converted to fatty acid methyl esters (FAMEs) by methanolsysis (transesterification reaction). Finally, FAMEs were analyzed on a Nexis GC-2030 gas chromatograph (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) to determine the types and quantities of microorganisms. PLFA and NLFA microbial abundance was reported in ng/g of soil.

Total microbial biomass is the sum of arbuscular mycorrhizal (AMF), saprophytic fungi, gram negative, gram positive, actinomycetes, protozoa, rhizobia, and undifferentiated biomass. Total bacteria biomass is the sum of the gram positive, gram negative, and actinomycetes. Total fungal biomass is based on the sum of AMF and saprophytic fungi. Fungi: bacteria ratio is based on the total fungi biomass to the total bacteria biomass.

The biomarkers used for microbial analysis were as follows: gram-negative bacteria 16:1 w7c, 18:1 w5c, 18:1 w7c; gram-positive bacteria 14:0 iso, 15:00, 15:0 iso, 15:0 anteiso, 16:0 iso, 17:00, 17:0 iso, 17:0 anteiso; arbuscular mycorrhizae 16:1 w5c; actinomycetes 16:0 10-methyl, 17:0 10-methyl, 18:0 10-methyl; saprophytic fungi 18:1 w9c, 18:2 w6c, 18:3 w6c, 20:5 w3c; protozoa 20:3 w6c; rhizobia 17:0 iso 3OH.
Undifferentiated biomarkers were 10:00, 12:00 anteiso, 13:00 iso, 13:00 anteiso, 13:1 w5c, 14:00, 14:1 w7c, 14:1 w9c, 15:1 w6c, 15:1 iso w6c, 15:1 anteiso w9c, 15:4 w3c, 16:0, 17:1 iso w9c, 17:1 anteiso w9c, 17:1 w8c, 17:0 cyclo w7c, 17:1 w7c 10-methyl, 17:1 w6c, 17:1 w7c, 17:1 w4c, 17:1 w5c, 18:0 iso, 18:0, 18:1 w7c 10-methyl, 19:0, 19:0 cyclo w7c, 19:1 w7c, 19:1 w7c 10-methyl, 19:1 w8c, 19:3 w6c, 19:4 w6c, 20:1 w6c, 20:0, 20:0 iso, 20:1 w8c, 21:0, 21:1 w3c, 21:1 w4c 21:1 w5c, 21:1 w8c, 22:1 w6c, 22:5 w6c, 22:6 w3c, 22:2 w6c, 22:0, 23:4 w6c, 23:0, 24:0, 24:1 w9c.

2.5 Statistical analysis

Statistical analyses were performed in R project (R Core team, 2023) and RStudio (Posit team, 2023) by using lmer in the package lme4 (Bates et al., 2015). A linear mixed-effects model ANOVA was fit to assess the effect of treatment (main factor), location and depth (nested withing each treatment block combination) on the Min C, POXC, soil aggregate size fractions, and total soil C (%). A linear mixed-effects model ANOVA was fit to assess the effect of treatment (main factor) and location on the PLFA and NLFA data, as well as total C, Min C and POXC in 2023. Fixed factors are treatment, location and depth, and the random factor is replication of the treatment (or blocks). The assumptions of normality and homogeneity of variance were tested using the Shapiro-Wilk test and Levene’s test. Data transformation was performed as needed to meet normality and homogeneity of variance assumptions. Tukey’s HSD test was used to compare data means for significant effects (p-value <0.05).
CHAPTER 3: RESULTS

3.1 Effects of cover crops and inoculation with AMF on soil C sequestration indicators

To assess the effects of cover cropping and inoculation with AMF on soil C dynamics, we performed analyses of soil total C, POXC, and Min C for soils collected two- and three-years following practice implementation. For soil collected 2 years following practice implementation, we also determined soil aggregation.

After two years of treatment implementation, there was no treatment effect on the total soil C (%) concentration; however, there was a significant location by depth interactive effect on total soil C (p<0.001) (Figure 3). The total soil C concentration was significantly greater in the topsoil of the alley row (2.7%) compared to the topsoil of the tree row (1.9%), the subsoil of the alley row (1.7 %), and the subsoil of the tree row (1.9 %).
Figure 3. Average total soil carbon (%) concentrations for the control, non-legume cover crop (NLCC) and non-legume cover crop inoculated with mycorrhizae (NLCC-M) treatments in middle of the alley row and the tree row in the 0-6 inch and 6–18-inch depth increments at the commercial lemon orchard representing 2 years post treatment establishment. Error bars represent standard errors (n = 3). Different lower-case letters indicate significant differences for depth by location interactions.

There was no treatment or location effect on POXC content, but POXC was significantly greater (p≤0.05) in the top 0 to 6 inch of soil (515 mg C kg soil⁻¹) than in the subsoil increment of 6 to 18 inches (477 mg C kg soil⁻¹) across treatments and locations (Figure 4). Similar to POXC, Min C was not impacted by the treatments; however, there was a significant location by depth interactive effect (p=0.001). Min C was significantly higher in the topsoil middle of the row location compared to the topsoil tree row and to the subsoil tree and row locations (Figure 5).
Figure 4. Average permanganate oxidizable carbon (POXC, mg C kg soil\(^{-1}\)) concentrations for the control, non-legume cover crop (NLCC) and non-legume cover crop inoculated with mycorrhizae (NLCC-M) treatments in middle of the alley row and the tree row in the 0-6 inch and 6–18-inch depth increments at the commercial lemon orchard representing 2 years post treatment establishment. Error bars represent standard errors (n = 3). Different upper-case letters indicate significant differences between depths.
Figure 5. Average mineralizable carbon concentrations (Min C, mg C kg soil\(^{-1}\) day\(^{-1}\)) for the control, non-legume cover crop (NLCC) and non-legume cover crop inoculated with mycorrhizae (NLCC-M) treatments in middle of the alley row and the tree row in the 0-6-inch and 6-18-inch depth increments at the commercial lemon orchard representing 2 years post treatment establishment. Error bars represent standard errors (n = 3). Different lower-case letters indicate significant differences between depth by location interactions.

The treatments had no effect on the Min C:POXC ratio. The Min C:POXC ratio was significantly higher (p<0.001) in the topsoil alley row compared to the topsoil tree row and the subsoil tree row indicating that soil respiration relative to labile C accumulation was greater in the topsoil alley row compared to the tree row (Figure 6). Within the subsoil, the Min C:POXC ratio was similar at both locations. Within the tree row or the alley row location, the Min C:POXC ratio was similar at both depths.
Figure 6. Min C to POXC ratio for the control, non-legume cover crop (NLCC) and non-legume cover crop inoculated with mycorrhizae (NLCC-M) treatments in middle of the alley row and the tree row in the 0-6-inch and 6-18-inch depth increments at the commercial lemon orchard representing 2 years post treatment establishment. Error bars represent standard errors (n = 3). Different lowercase letters indicate significant differences between depths and locations in case of a significant depth by location interaction.

There was no treatment effect on aggregate size distribution after two years of treatment implementation (Table 1). For large macroaggregates, location influenced soil aggregate stability depending on the depth (p≤0.01). The large macroaggregate fraction was significantly greater in the topsoil middle of the row compared to the area next to the tree across all treatments. The small macroaggregate fraction was significantly greater in the middle of the row (mean of 54.8%) compared to the area next to the tree (mean of 48.5%), while the silt and clay fraction was significantly lower in middle of the row (mean of 7.8%) compared to the area next to the tree (mean of 9.1%) across depths and treatments. The microaggregate fraction in the topsoil was significantly lower in middle of the row (mean 27.5%) than the area next to the tree (mean 40.8%) but not different
compared to the subsoil at both locations (mean for the tree 32.5 % and row 30.7%), and the topsoil next to the tree fraction was significantly greater than the subsoil middle of the row.
Table 1. Mean aggregate size distribution and the respective standard errors of the mean (n = 3) for the control, non-legume cover crop (NLCC) and non-legume cover crop inoculated with mycorrhizae (NLCC-M) treatments in middle of the alley row and the tree row in the 0-6-inch and 6-18-inch depth increments at the commercial lemon orchard representing 2 years post treatment establishment. Soil aggregate fractions were large macroaggregates (> 2mm), small macroaggregates (2 mm - 250 μm), microaggregates (250 μm -53 μm), and the silt and clay fraction (< 53 μm). Significant differences of main effects of location are shown as different uppercase letters within the same aggregate size; different lowercase letters indicate significant differences between depths and locations in case of a significant depth by location interaction within the same aggregate size.

<table>
<thead>
<tr>
<th></th>
<th>Large macroaggregates (%)</th>
<th>Small macroaggregates (%)</th>
<th>Microaggregates (%)</th>
<th>Silt and clay (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Row</td>
<td>Tree</td>
<td>Row</td>
<td>Tree</td>
</tr>
<tr>
<td>0-6 in</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>20.7 ± 8.5</td>
<td>3.6 ± 0.8</td>
<td>51.9 ± 3.4</td>
<td>47.6 ± 6.3</td>
</tr>
<tr>
<td>NLCC</td>
<td>7.9 ± 3.2</td>
<td>2.6 ± 0.8</td>
<td>57.4 ± 4.0</td>
<td>46.4 ± 4.8</td>
</tr>
<tr>
<td>NLCC-M</td>
<td>7.5 ± 2.1</td>
<td>3.5 ± 0.5</td>
<td>49.3 ± 2.1</td>
<td>45.9 ± 2.9</td>
</tr>
<tr>
<td>6-18 in</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3.8 ± 1.2</td>
<td>11.7 ± 8.4</td>
<td>51.6 ± 5.0</td>
<td>49.1 ± 3.3</td>
</tr>
<tr>
<td>NLCC</td>
<td>4.6 ± 1.3</td>
<td>8.3 ± 4.3</td>
<td>63.0 ± 2.6</td>
<td>56.7 ± 3.9</td>
</tr>
<tr>
<td>NLCC-M</td>
<td>4.5 ± 1.8</td>
<td>4.2 ± 0.5</td>
<td>55.6 ± 5.1</td>
<td>45.4 ± 2.3</td>
</tr>
</tbody>
</table>
Table 2. Three-way ANOVA of the total soil carbon, Min C, POXC, MinC:POXC ratio, and percent soil in each aggregate fraction by treatment, functional location and depth at the citrus orchard post second season treatment application. Soil aggregate fractions were large macroaggregates (LM), small macroaggregates (SM), microaggregates (m), and the silt and clay fraction (S+C). F and p represent F-value and p value, respectively.

<table>
<thead>
<tr>
<th></th>
<th>Total soil C</th>
<th>Min C</th>
<th>POXC</th>
<th>MinC:POXC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>p</td>
<td>F</td>
<td>p</td>
</tr>
<tr>
<td>Treatment</td>
<td>0.29</td>
<td>0.758</td>
<td>0.62</td>
<td>0.544</td>
</tr>
<tr>
<td>Location</td>
<td>16.19</td>
<td>0.001</td>
<td>16.60</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Depth</td>
<td>51.20</td>
<td>0.000</td>
<td>1.37</td>
<td>0.253</td>
</tr>
<tr>
<td>Treatment:Location</td>
<td>0.64</td>
<td>0.540</td>
<td>1.33</td>
<td>0.282</td>
</tr>
<tr>
<td>Treatment:Depth</td>
<td>1.02</td>
<td>0.381</td>
<td>0.23</td>
<td>0.797</td>
</tr>
<tr>
<td>Location:Depth</td>
<td>53.88</td>
<td>&lt;0.001</td>
<td>15.94</td>
<td>0.001</td>
</tr>
<tr>
<td>Treatment:Location:Depth</td>
<td>0.74</td>
<td>0.493</td>
<td>0.04</td>
<td>0.960</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>LM</th>
<th>SM</th>
<th>m</th>
<th>S+C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>p</td>
<td>F</td>
<td>p</td>
</tr>
<tr>
<td>Treatment</td>
<td>0.69</td>
<td>0.538</td>
<td>3.36</td>
<td>0.105</td>
</tr>
<tr>
<td>Location</td>
<td>2.54</td>
<td>0.129</td>
<td>7.49</td>
<td>0.014</td>
</tr>
<tr>
<td>Depth</td>
<td>0.40</td>
<td>0.534</td>
<td>2.78</td>
<td>0.113</td>
</tr>
<tr>
<td>Treatment:Location</td>
<td>0.04</td>
<td>0.957</td>
<td>0.45</td>
<td>0.642</td>
</tr>
<tr>
<td>Treatment:Depth</td>
<td>0.63</td>
<td>0.544</td>
<td>0.89</td>
<td>0.429</td>
</tr>
<tr>
<td>Location:Depth</td>
<td>8.57</td>
<td>0.009</td>
<td>0.00</td>
<td>0.977</td>
</tr>
<tr>
<td>Treatment:Location:Depth</td>
<td>0.82</td>
<td>0.458</td>
<td>0.55</td>
<td>0.584</td>
</tr>
</tbody>
</table>

Table 3. Three-way ANOVA of the total soil carbon, Min C, POXC, and MinC:POXC by treatment and functional location at the citrus orchard post third season treatment application. F and p represent F-value and p value, respectively.

<table>
<thead>
<tr>
<th></th>
<th>Total soil C</th>
<th>Min C</th>
<th>POXC</th>
<th>MinC:POXC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>p</td>
<td>F</td>
<td>p</td>
</tr>
<tr>
<td>Treatment</td>
<td>0.06</td>
<td>0.942</td>
<td>0.13</td>
<td>0.877</td>
</tr>
<tr>
<td>Location</td>
<td>49.38</td>
<td>&lt;0.001</td>
<td>39.78</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Treatment:Location</td>
<td>0.39</td>
<td>0.877</td>
<td>0.74</td>
<td>0.627</td>
</tr>
</tbody>
</table>

After three years of treatment implementation, total soil C ranged from 1.1 % to 2.4 % at Location 1 (on top of the berm next to the tree), from 1.3 % to 2.8 % at Location 2 (in the fallow area next to the berm), from 1.5 % to 3.2 % at Location 3 (on the fringe of the cover crop area), and from 1.9 % to 3.3 at Location 4 (in the middle of the cover crop area) (Figure 7). There was no significant effect of treatments on the total soil C (%).
across the four functional locations, but the effect of location on the total soil C was significant (p<0.001). The total soil C content was significantly higher in Locations 3 and 4 than in Locations 1 and 2 and in Location 2 compared to Location 1.

![Figure 7](image)

Figure 7. Mean total soil carbon (%) concentrations for the control, self-germinated non-legume cover crop (NLCC) and self-germinated non-legume cover plus effect of three-years of AMF inoculation (NLCC-M) treatments in 4 functional locations ((1) on top of the berm next to the tree, (2) in the fallow area next to the berm, (3) on the fringe of the cover crop area, and (4) in the middle of the cover crop area) in the 0-6 inch depth increment at the commercial lemon orchard after 3 years of treatments implementation. Error bars represent standard errors (n = 3). Different upper-case letters indicate significant differences between locations.

Average permanganate oxidizable carbon (POXC, mg C kg soil\(^{-1}\)) was similar across Control, NLCC, and NLCC-M treatments (Figure 8). POXC concentration was significantly higher (p<0.001) in Location 4 compared to Locations 1 and 2. Location 1 had the lowest POXC content, which was significantly different from all other locations. Overall, POXC concentration ranged from 245.8 to 557.9 mg C kg soil\(^{-1}\).
Figure 8. Average permanganate oxidizable carbon (POXC, mg C kg soil\(^{-1}\)) concentrations for the control, self-germinated non-legume cover crop (NLCC) and self-germinated non-legume cover plus effect of three-years of AMF inoculation (NLCC-M) treatments in 4 functional locations ((1) on top of the berm next to the tree, (2) in the fallow area next to the berm, (3) on the fringe of the cover crop area, and (4) in the middle of the cover crop area) in the 0-6 inch depth increment at the commercial lemon orchard after 3 years of treatments implementation. Error bars represent standard errors (n = 3). Different upper-case letters indicate significant differences between locations.

Average mineralizable carbon concentrations (Min C, mg C kg soil\(^{-1}\) day\(^{-1}\)) followed a similar trend as POXC. Min C was similar across Control, NLCC, and NLCC-M treatments (Figure 9), but the effect of location on the Min C concentration was significant (p<0.001). Min C concentration was higher in Locations 3 and 4 compared to the Locations 1 and 2. Overall, Min C ranged from 8.3 to 53.6 mg C kg soil\(^{-1}\) day\(^{-1}\).
Figure 9. Average mineralizable carbon concentrations (Min C, mg C kg soil$^{-1}$ day$^{-1}$) for the control, self-germinated non-legume cover crop (NLCC) and self-germinated non-legume cover plus effect of three-years of AMF inoculation (NLCC-M) treatments in 4 functional locations ((1) on top of the berm next to the tree, (2) in the fallow area next to the berm, (3) on the fringe of the cover crop area, and (4) in the middle of the cover crop area) in the 0-6 inch depth increment at the commercial lemon orchard after 3 years of treatments implementation. Error bars represent standard errors (n = 3). Different uppercase letters indicate significant differences between locations.

There was no treatment effect on Min C:POXC ratio, but the effect of location on the Min C:POXC ratio was significant (p<0.001) (Figure 10). The Min C:POXC ratio was significantly higher in Locations 3 and 4 compared to Locations 1 and 2. Increases in the Min C:POXC ratio indicates that there was more soil respiration relative to labile C accumulation in Locations 3 and 4 (Figure).
Figure 10. Min C to POXC ratio for the control, self-germinated non-legume cover crop (NLCC) and self-germinated non-legume cover plus effect of three-years of AMF inoculation (NLCC-M) treatments in 4 functional locations ((1) on top of the berm next to the tree, (2) in the fallow area next to the berm, (3) on the fringe of the cover crop area, and (4) in the middle of the cover crop area) in the 0-6 inch depth increment at the commercial lemon orchard after 3 years of treatments implementation. Error bars represent standard errors (n = 3). Different upper-case letters indicate significant differences between locations.

3.2 Impact of inoculation with AMF, cover crop, and location on the soil microbial community

The effect of cover crops, inoculation with AMF and location on the microbial community was assessed based on PLFA and NLFA analysis of soil samples collected in February 2023, representing 3 years of treatment implementation. There was no treatment effect on assessed microbial groups (Table 4). Total soil microbial biomass was influenced by location (p<0.001) with the highest biomass in the middle of the cover crop row (Location 4) and lower biomass in the berm near the trees and the fallow area next to the berm (Locations 1 and 2). Location had a significant (p<0.001) effect on the total
fungi biomass, arbuscular mycorrhizal fungi (AMF) biomass, and saprophytic fungi biomass for PLFA as well as NLFA biomarkers. The PLFA total fungi biomass was highest in the middle of the cover crop and the fringe (edge) (Locations 3 and 4) compared to the fallow area next to the berm and the berm (Locations 1 and 2), and the NLFA total fungi biomass was the highest in the middle of the cover crop with a gradual fungal biomass decrease towards the berm (Location 1). Both PLFA and NLFA biomarkers for AMF were found across all four functional locations and all treatments. The AMF biomass PLFA was significantly different across four locations with the highest AMF biomass in the middle of the cover crop and the lowest in the berm. The NLFA AMF biomass was higher in the middle of the cover crop area compared to the other three locations. The PLFA saprophytic fungi biomass was the highest in the middle of the cover crop and the fringe (edge), lower in the fallow area next to the berm, and the lowest in the berm. The NLFA saprophytic fungi biomass had a different pattern across the four locations with the berm having the lowest biomass compared to the other three locations that were not statistically different among themselves.

The PLFA of total bacterial, gram-positive, and actinomycetes biomass showed the same distribution pattern. The PLFA total bacteria, gram-positive, and actinomycetes biomass was significantly (p<0.001) higher in the middle of the cover crop row (Location 4) compared to all other locations. The PLFA total bacteria, gram-positive, and actinomycetes biomass was also significantly greater in the fringe location (Location 3) compared to the fallow next to the berm and the berm location, while the latter two locations (Locations 1 and 2) were not different from each other. The PLFA gram negative biomass was significantly different across all four locations with the middle of
the cover crop (Location 4) being the highest and the berm near the trees (Location 1) the lowest.

Location had a significant (p<0.05) effect on the AMF:Saprophytes PLFA ratio but no effect on the NLFA ratio. The AMF:Saprophytes PLFA ratio was significantly greater in the center of the cover crop (location 4) with a gradual decrease towards the berm (Location 1). The total fungi to bacteria PLFA ratio was significantly (p<0.001) lower in the berm (Location 4) compared to the other three locations that were not statistically different among themselves (Locations 1, 2, and 3). The gram positive to gram negative ratio was significantly (p<0.001) greater in the center of the cover crop (Location 4) compared to the berm and the fallow area next to the berm (Locations 1 and 2) and lower in the berm compared to the fallow area next to the berm (Location 2) and the edge of the cover crop (Location 3) that were not statistically difference among themselves (Locations 2 and 3).
Table 4. Mean abundance of microbial groups (ng/g soil) and the respective standard errors of the mean (n = 3) for the control, non-legume cover crop (NLCC) and non-legume cover crop inoculated with mycorrhizae (NLCC-M) treatments in in 4 functional locations ((1) on top of the berm next to the tree, (2) in the fallow area next to the berm, (3) on the fringe of the cover crop area, and (4) in the middle of the cover crop area) in the 0-6 inch depth increment at the commercial lemon orchard after 3 years of treatments implementation. The microbial abundance is based on PLFA and NLFA biomarkers. Significant differences of main effects of location withing a microbial group are shown as different uppercase letters.

<table>
<thead>
<tr>
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<th>Location 1</th>
<th>Location 2</th>
<th>Location 3</th>
<th>Location 4</th>
</tr>
</thead>
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<tr>
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<td>(C)</td>
<td>(B)</td>
<td>(A)</td>
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<tr>
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<tr>
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<td>(B)</td>
<td>(A)</td>
<td>(A)</td>
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<tr>
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<td>334.5±60.6</td>
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<td>(A)</td>
<td>(A)</td>
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<tr>
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<td>1004.5±150.3</td>
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<td>NLCC-M</td>
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<td>647.8±140.2</td>
<td>1004.3±345.8</td>
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<td>(B)</td>
<td>(A)</td>
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<td>(A)</td>
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<td>Location 3</td>
<td>Location 4</td>
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<td>------------</td>
<td>------------</td>
<td>------------</td>
</tr>
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<td>Gram-positive</td>
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<td>(C)</td>
<td>(B)</td>
<td>(A)</td>
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<td>Control</td>
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<td>(C)</td>
<td>(B)</td>
<td>(A)</td>
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<td>AMF: Saprophytes</td>
<td>(B)</td>
<td>(AB)</td>
<td>(AB)</td>
<td>(A)</td>
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<td>Control</td>
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<td>(A)</td>
<td>(A)</td>
<td>(A)</td>
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<td>(AB)</td>
<td>(B)</td>
<td>(C)</td>
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<td>Control</td>
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Table 4 continued.

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<th></th>
<th>NLFA (ng/g soil)</th>
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<th>Location 3</th>
<th>Location 4</th>
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<tbody>
<tr>
<td><strong>Total Fungi Biomass</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>(C)</td>
<td>323.7±92.8</td>
<td>795.2±158.4</td>
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<td>1832.2±202.2</td>
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<td><strong>AMF</strong></td>
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<tr>
<td>Control</td>
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</table>

3.3 **Relationship among microbial groups and C cycling indicators**

Based on the PLFA biomarkers, microbial groups had a positive correlation among each other (Table 5). AMF biomass PLFA showed a strong positive correlation (p<0.001) with gram-negative bacteria, gram-positive bacteria biomass, and actinomycetes, and AMF biomass NLFA showed a significant (p<0.01) positive correlation with the same bacterial groups. AMF biomass PLFA was more positively correlated with saprophyte fungi biomass PLFA (p<0.001) than NLFA (p<0.05), and AMF biomass NLFA was positively correlated with saprotrophic fungi biomass PLFA (p<0.001) and showed no correlation with saprotrophic fungi biomass NLFA. All bacterial groups were positively correlated among each other. Saprophytic fungi biomass
PLFA was positively correlated with all microbial groups, but biomass NLFA showed no correlation with actinomycetes biomass.

Table 5. Pearson’s correlations between AMF biomass PLFA or NLFA, saprophytic fungi PLFA or NLFA, total bacteria biomass, gram-negative (G-) bacteria, gram-positive (G+) bacteria, and actinomycetes biomass. PLFA (ng/g soil) and NLFA (ng/g soil) are phospholipid fatty acids and neutral lipid fatty acids, respectively.

<table>
<thead>
<tr>
<th>Total bacteria</th>
<th>Act</th>
<th>G-</th>
<th>G+</th>
<th>Sap PLFA</th>
<th>Sap NLFA</th>
<th>AMF PLFA</th>
<th>AMF NLFA</th>
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<td>1.00***</td>
<td>1.00***</td>
<td>0.89***</td>
<td>0.39*</td>
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<td>Act</td>
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<td>0.95***</td>
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<td>G-</td>
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<td>0.89***</td>
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<td>G+</td>
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<td>0.37*</td>
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</table>

AMF- arbuscular mycorrhizal fungi; Sap- saprophytic fungi; Act- actinomycetes.
*Significant at the 0.05 probability level.
**Significant at the 0.01 probability level.
***Significant at the 0.001 probability level.
NS – not significant.

In order to investigate the influence of microbial groups on soil C dynamics, correlation analyses were performed between AMF biomass based on PLFA and NLFA biomarkers, total bacteria based on PLFA biomarkers, and saprophytes biomass based on PLFA and NLFA markers vs. Min C, POXC, total soil C and Min C:POXC ratio (Table 6). AMF biomass PLFA had a positive correlation with total soil C, POXC, Min C, and MinC:POXC ratio. AMF biomass NLFA had only a positive correlation with the MinC:POXC ratio. Total bacterial biomass PLFA and saprophyte biomass PLFA had similar correlation trends as AMF biomass PLFA. Saprophytes biomass NLFA was positively correlated with Min C and the MinC:POXC ratio.
Table 6. Pearson’s correlation between total bacterial biomass PLFA, AMF and saprophytic fungi biomass PLFA or NLFA and Min C (mg C kg soil$^{-1}$ day$^{-1}$), POXC (mg C kg soil$^{-1}$), Min C:POXC ratio, and total soil carbon (%).

<table>
<thead>
<tr>
<th>Total soil C (%)</th>
<th>POXC</th>
<th>Min C</th>
<th>Min C:POXC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total bacteria</td>
<td>0.57***</td>
<td>0.64***</td>
<td>0.86***</td>
</tr>
<tr>
<td>Sap PLFA</td>
<td>0.39*</td>
<td>0.50**</td>
<td>0.74***</td>
</tr>
<tr>
<td>Sap NLFA</td>
<td>0.31NS</td>
<td>0.32NS</td>
<td>0.53***</td>
</tr>
<tr>
<td>AMF PLFA</td>
<td>0.48**</td>
<td>0.61***</td>
<td>0.83***</td>
</tr>
<tr>
<td>AMF NLFA</td>
<td>0.07NS</td>
<td>0.08NS</td>
<td>0.32NS</td>
</tr>
</tbody>
</table>

PLFA (ng/g soil) and NLFA (ng/g soil) are phospholipid fatty acids and neutral lipid fatty acids, respectively. AMF - arbuscular mycorrhizal fungi. Sap - saprophytic fungi.

*Significant at the 0.05 probability level.
**Significant at the 0.01 probability level.
***Significant at the 0.001 probability level.

NS – not significant

3.4 Soil chemical and physical properties across orchard floor

Soil chemical and physical properties such as pH, soil salinity (EC, dS/m), and soil moisture content (%) were measured three years after treatment implementation (Table 7). There was no influence of treatment or location on soil pH or EC. Average soil pH ranged from 8.10 to 8.51 across the samples and average EC ranged from 2.51 to 3.55 dS/m. Soil moisture (%) was significantly lower (p<0.001) in Location 1 compared to the Locations 2, 3, and 4 at the time of sampling.
Table 7. Average (values ± SD, n=3) pH, electrical conductivity (EC, dS/m), and soil moisture content (%) for the control, non-legume cover crop (NLCC) and non-legume cover crop inoculated with mycorrhizae (NLCC-M) treatments in 4 functional locations ((1) on top of the berm next to the tree, (2) in the fallow area next to the berm, (3) on the fringe of the cover crop area, and (4) in the middle of the cover crop area) in the 0-6 inch depth increment at the commercial lemon orchard after 3 years of treatments implementation. Different upper-case letters indicate significant differences between locations.

<table>
<thead>
<tr>
<th>Location</th>
<th>pH (-)</th>
<th>EC (dS/m)</th>
<th>Moisture (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>NLCC</td>
<td>NLCC-M</td>
</tr>
<tr>
<td>pH (-)</td>
<td>8.37 ± 0.13</td>
<td>8.51 ± 0.13</td>
<td>8.47 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>8.39 ± 0.14</td>
<td>8.34 ± 0.14</td>
<td>8.10 ± 0.29</td>
</tr>
<tr>
<td></td>
<td>8.39 ± 0.18</td>
<td>8.41 ± 0.11</td>
<td>8.32 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>8.33 ± 0.13</td>
<td>8.35 ± 0.20</td>
<td>8.23 ± 0.14</td>
</tr>
<tr>
<td>EC (dS/m)</td>
<td>Control</td>
<td>NLCC</td>
<td>NLCC-M</td>
</tr>
<tr>
<td></td>
<td>2.60 ± 0.20</td>
<td>2.60 ± 0.14</td>
<td>2.51 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>2.90 ± 0.24</td>
<td>2.83 ± 0.26</td>
<td>3.55 ± 0.92</td>
</tr>
<tr>
<td></td>
<td>2.98 ± 0.29</td>
<td>2.78 ± 0.23</td>
<td>3.01 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>2.89 ± 0.27</td>
<td>2.95 ± 0.32</td>
<td>2.96 ± 0.18</td>
</tr>
<tr>
<td>Moisture (%)</td>
<td>Control</td>
<td>NLCC</td>
<td>NLCC-M</td>
</tr>
<tr>
<td>(B)</td>
<td>0.24 ± 0.03</td>
<td>0.25 ± 0.01</td>
<td>0.23 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>0.28 ± 0.03</td>
<td>0.27 ± 0.01</td>
<td>0.27 ± 0.02</td>
</tr>
<tr>
<td>(A)</td>
<td>0.30 ± 0.03</td>
<td>0.27 ± 0.03</td>
<td>0.28 ± 0.02</td>
</tr>
<tr>
<td>(A)</td>
<td>0.31 ± 0.05</td>
<td>0.27 ± 0.02</td>
<td>0.27 ± 0.02</td>
</tr>
</tbody>
</table>

Table 8. Three-way ANOVA of pH, Min C, EC (dS/m), and soil moisture (%) by treatment and functional location at the citrus orchard post third season treatment application. F and p represent F-value and p value, respectively.

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>EC</th>
<th>% Moisture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>p</td>
<td>F</td>
</tr>
<tr>
<td>Treatment</td>
<td>0.27</td>
<td>0.769</td>
<td>0.13</td>
</tr>
<tr>
<td>Location</td>
<td>1.35</td>
<td>0.291</td>
<td>2.56</td>
</tr>
<tr>
<td>Treatment:Location</td>
<td>0.51</td>
<td>0.790</td>
<td>0.28</td>
</tr>
</tbody>
</table>
CHAPTER 4: DISCUSSION

3.1 Effects of cover crops and inoculation with AMF on soil C sequestration indicators

After two years of treatment implementation, there was no effect of cover crops or inoculation with arbuscular mycorrhizal fungi (AMF) on the soil total C, permanganate oxidizable carbon (POXC), mineralizable carbon (Min C) and soil aggregation in the tree or alley row at 0-6 inches or 6-18 inches depth (Figures 3-6, Table 1). Likewise, there was no treatment effect on total soil C, POXC, Min C, and the Min C:POXC ratio at any of the four locations across the orchard floor at 0-6 inches depth after three years of treatment implementation (Figures 7-9). These findings oppose our hypothesis that the cereal cover crop and inoculation with arbuscular mycorrhizal fungi (AMF) would increase total soil C compared to fallowed orchard alleys.

Several pot and chamber studies focusing on inoculation with AMF showed a positive effect of AMF on soil organic carbon (SOC) (Fang et al., 2020; J. L. Hu et al., 2014; Subramanian et al., 2019; Y.-J. Wang et al., 2023). In a chamber experiment with Trifoliate orange seedlings used as AMF host, Wang et al. (2023) found that under adequate watering, AMF (*Rhizophagus intraradices*) mycelia increased SOC compared to the non-inoculated plants. Likewise, a 5-month field study showed a positive effect of AMF inoculation (*Funneliformis mosseae*) on SOC by inoculating wild cherry, cerasus humilis, apricot, shiny leaf yellow horn tree seedlings (Z.-G. Wang et al., 2016). In a field study with maize in a semiarid climate, plots inoculated with AMF (*Funneliformis mosseae*) showed no effect on total soil C, but increased labile organic C pools including easily oxidizable organic C, particulate organic C and, light fraction organic C compared to treatments with plastic film, no plastic film and no AMF inoculation, and plastic film
with AMF inoculation after two years of treatment implementation (Ren et al., 2021). Soil aggregation plays an important role in C sequestration due to physical protection of soil organic matter from degradation. A five-week laboratory incubation experiment using Plantago lanceolata as a host, AMF inoculum, and rare earth elements as tracking tools showed that AMF had a stabilizing effect on large macroaggregates and large macroaggregates formed faster with AMF (Morris et al., 2019). A positive correlation was found between native AMF and large macroaggregates under various field management types (Lin et al., 2023; Wilson et al., 2009).

The lack of the effect of the inoculated cover crop compared to the non-inoculated cover crop and control treatment on measured soil C metrics in our study may be attributed to native AMF species forming associations with plants growing in the alley row and leading to similar results as those observed in the inoculated plots. Effect of AMF inoculation on various metrics is predominantly measured in greenhouse studies under controlled conditions with sterilized controls; thus, effects of AMF inoculation may be greater in greenhouse studies compared to field studies where control treatments have native AMF propagules (Berruti et al., 2016). In order to achieve non-mycorrhizal controls, fumigation of soil can be used to suppress native AMF (Ren et al., 2021; Sylvia et al., 1993) or plants with reduced AMF colonization (Bowles, Barrios-Masias, et al., 2016). However, constant fumigation of treatment plots within alley rows would be difficult to achieve in the orchard; thus, there was no native AMF suppression in this orchard trial. Pérez-Guzmán et al. (2021) observed an increase of native AMF under grass cover crop compared to corn or soybean crops, and late cover crop termination increased native AMF abundance compared to a fallow in dryland cropping system (H.
Likewise, a meta-analysis showed that cover cropping promotes native AMF abundance leading to better AMF colonization of cash crops (Bowles, Jackson, et al., 2016). Zero tillage with a grass cover (Cenchrus ciliaris) and maize grown on a raised bed showed the highest native AMF abundance and increase in soil C compared to conventional tillage with soybean-wheat rotation (Agnihotri et al., 2021). A positive correlation of native AMF abundance and soil C was observed in native multispecies prairie communities (Wilson et al., 2009) and in a no till treatment with field crops (Lin et al., 2023). Similarly, native AMF communities and their effects on soil C may have been promoted by the cover crop in our study. Likewise, the control treatment had a lot of weed growth, which was dominated by Malva, leading to the presence of plants in the control plots. No herbicide was applied in the alley rows of the control treatment following the typical grower practice at this site. It was found that certain weeds have the potential to promote AMF in soil. For example, in a citrus orchard study, natural grass cover with weeds present across the entire orchard floor increased native AMF propagules, citrus root colonization by AMF, and soil organic carbon compared to clean tillage in soil collected from the outermost circumference of the tree canopy (P. Wang et al., 2016a). Our findings suggest that native AMF can be successfully promoted in the alley rows by weeds or a cereal cover crop and that AMF inoculation may not be necessary to promote AMF in perennial citrus orchards.

3.2 Effects of orchard floor location on soil C sequestration indicators

In contrast to treatment comparisons, soil C indicators showed greater values in the alley rows compared to the tree row. A positive effect of different cover crops on soil C compared to tilled or fallow controls across alley rows were observed in many orchard
studies (Castellano-Hinojosa et al., 2023; Guimarães et al., 2013; Q. Hu et al., 2023; Márquez-García et al., 2024; Ramos et al., 2010; Steenwerth & Belina, 2008; Visconti et al., 2022); however, the studies that measured soil C dynamics in both the tree rows and alley rows are limited. In this lemon orchard trial, soils in the tree rows and the alley rows were under different management systems. The alley rows had a presence of cover crop or weeds and received mulch (woodchips) from tree clippings (canopy pruning) every year, while soil in the tree rows was kept bare and organic matter additions were limited to tree leaf litter. The tree rows also were subjected to more dry-wet cycles during irrigation in dry months, which coincides with the growing season in California’s climate (April-October). Plant root residues and above ground plant litter contribute to soil organic matter (SOM) content (Austin et al., 2017; Kong & Six, 2010; Weil & Brady, 2009), and across multiple studies summarized in a meta-analysis and a review papers, cover crops were shown to have a positive impact on the soil C content (Blanco-Canqui et al., 2015; McClelland et al., 2021). Besides cover crop contribution to SOM, shredded tree clippings applied to soil as mulch can contribute to SOM as well. In soil restoration studies, woodchips, compared to control, were found to increase soil organic C in heavy disturbed soils (Eldridge et al., 2012; Espinosa et al., 2020). Pecan wood chips increased soil organic matter, compared to control, at a higher rate and after repeated additions (Tahboub et al., 2008). Likewise, grapevine prunings increased soil organic C in a vineyard trial (Yilmaz et al., 2017) and willow chips increased total C in an annual cropping system compare to a non-amended control (Uwituze et al., 2023). The tree rows in the irrigated orchards often experience different carbon inputs because the topsoil is often kept bare (plant free). Citrus trees’ bulk of the roots are located within the footprint
of the canopy, especially around drip emitters (Morgan et al., 2007); thus, in the tree row, roots contribute to the soil C pool. Due to irrigation of the tree rows in dry months, repeated dry-wet cycles can lead to soil organic carbon loss. It was observed that freshly wetted soil can release CO2 because of a sudden pulse of C mineralization often associated with microbial activity (Denef et al., 2001; Lopez-Sangil et al., 2018; Lundquist et al., 1999). In a furrow irrigated orange orchard, frequent wet-dry cycles in the furrows created distinct soil conditions from the berm with trees leading to a highly heterogeneous pattern across the orchard floor (Avila et al., 2023).

Differences in management systems between the two locations were reflected in our soil C indicators data set with soil total C and Min C being higher in the topsoil in the cover cropped alley row compared to the tree row. A similar trend was observed in a pecan orchard study, where total soil C was higher in the alley row with cover crop compared to the tree row with bare soil (Rodriguez-Ramos et al., 2022). Likewise, POXC was impacted by the location management system with higher values observed in the topsoil in the alley row after 1 year (data is not shown) and 3 years (Figure 8); however, after the second year, POXC was not significantly different between the two functional locations (Figure 4). The lack of significant difference in POXC between the two locations after the second year reflects the sensitivity of POXC to capture short-term and dynamic changes in labile soil C. Even though Location 2, the area next to the berm, was plant free, total soil C and POXC in that location were similar to that on the edge of the cover crop (Location 3) suggesting a potential carry over effect from the cover crop into Location 2. Location also had an effect on aggregate size distribution with more large and small macroaggregates and less microaggregates in the alley rows compared to the
tree rows (Table 1). Soil aggregates play an important role in soil C sequestration by physically protecting SOM from biological degradation (Ramesh et al., 2019). Soil microaggregates can be found inside soil macroaggregates (Miller & Jastrow, 2000; Six et al., 2000, 2002); thus, macroaggregates are often associated with higher soil C sequestration (Lin et al., 2023; Wilson et al., 2009). The management in the alley rows more positively impacted soil C cycling indicators and soil health with some carry over from the cover cropped alley rows into adjacent areas. Location effect in this orchard trial suggest that different managent of trees and alley rows leads to soil heterogeneity across the orchard floor.

3.3 Impact of inoculation with AMF, cover crop, and location on the soil microbial community

Successful establishment of introduced AMF depends on multiple factors such as compatibility of introduced AMF with physical conditions of the soil and with plant genotype, quality of introduced AMF, formulation, and type of inoculants, frequency of application, soil biotic conditions, diversity and abundance of native AMF, and niche availability for introduced AMF (Basiru & Hijri, 2022; Sato et al., 2018, 2021; Verbruggen et al., 2012). In addition, high diversity of native AMF can outcompete and suppress introduced AMF, while low diversity and abundance of native AMF allows for better establishment of introduced AMF (Bender et al., 2019; Rodriguez & Sanders, 2015). In their review of ecological impacts of commercial AMF inoculants on native AMF, Basiru and Hijri (2022) observed that introduced AMF can suppress, stimulate, exclude or have no effect on native AMF. In our trial, we did not measure establishment and persistence of introduced AMF, so we cannot confirm whether inoculation of commercial *Rhizophagus intraradices* was successful or not. However, we evaluated
community structure on a broad scale across the entire orchard floor by performing
phospholipid fatty acid (PLFA) and neutral lipid fatty acid (NLFA) analysis on soils
collected during active growth of the cover crop (Figure 2). At the time of soil sampling,
the cover crop was self-seeded, and we did not inoculate with AMF inoculum; thus,
PLFA and NLFA biomass represents legacy effects of three years of cover crop planting
and inoculation with commercial inoculum of *Rhizophagus intraradices*. Our PLFA and
NLFA microbial biomass data showed no significant persistent inoculation impact on the
microbial community compared to the non-inoculated cover crop and the weedy control
treatment plots after three years of treatment implementation (Table 4). Moreover, there
was no difference in the microbial community between the non-inoculated cover crop
treatment and the control. We hypothesized that AMF, bacterial, and saprophytic fungal
biomass would be higher in treatments with cover crop compared to the control treatment
because the cover crop provides microorganisms with carbon sources such as root
exudates, residues, and plant biomass required for their function (Gilmullina et al., 2023;
Lehman et al., 2015). Our control treatment had weeds (predominantly *Malva* and some
filarees during soil sampling) which added carbon sources to soil, likely causing the lack
of a significant difference between cover crop treatments and control. Regarding AMF
biomass, because up to 74% of all plant species can form associations with AMF from
the Glomeromycota clade (Brundrett, 2009; Smith & Read, 2008) and several plant
species from Malvaceae were found to be colonized with AMF (Akhmetzhanova et al.,
2012; B. Wang & Qiu, 2006), there was a high potential that weeds in the control plots
formed association with native AMF resulting in similar abundance of PLFA and NLFA
AMF biomass between the control and the cereal cover crop. Different plant species
have been shown to promote specific microbial groups, for example, greater PLFA abundance of saprophytic fungi was observed under an oat cover crop compared to a rye cover crop in a field under organic management, while NLFA AMF abundance was similar in both the oat and rye cover crop treatments (Martínez-García et al., 2018). In a cover crop study using 8 different cover crop species (sunn hemp, soybean, red clover, hairy vetch forage radish, oat, canola, cereal rye), PLFA AMF biomass was higher in oat, rye, 8 species mix, and red clove+ hairy vetch+ forage radish +oat mix compared to an untilled weedy fallow control. In the same study, PLFA saprophytic fungi biomass was higher in hairy vetch, and PLFA gram negative (G-) biomass was higher in canola and 8 species mix compared to the control (Finney et al., 2017). In a field study with a winter wheat-sorghum-cover crop (fallow for control) rotation under no till, an oat cover crop and a diverse cover crop mix (pea, oat, canola, hairy vetch, forage radish, and barley) increased AMF and saprophytic fungi biomass compared to the fallow control (Thapa et al., 2021). In addition, several plant species that are considered agricultural weeds (velvetleaf, common lambsquarters, redroot pigweed, green foxtail, Digitaria sanguinalis (grass), Echinochloa crusgalli (grass), Acalypha australis (dicot), Portulaca oleracea (dicot), and Chenopodium album (dicot)) were found to have negative impacts on the microbial biomass (Finney et al., 2017; Wortman et al., 2013) and diversity (C. Yang et al., 2022). In our trial, we did not observe any differences in microbial biomass between the cereal cover crop and the weedy control treatment, indicating that weeds supported the microbial community similarly as the cereal cover crop. These findings support our suggestion that native AMF can be successfully promoted in the alley rows by weeds or a
cover crop, and may provide a more effective strategy than inoculation in perennial citrus orchards.

We also expected that the effect of the cover crop on the microbial community would decrease from the center of the alley row to the tree row, following patterns in plant inputs. Our results were consistent with our expectations showing a significant decrease of microbial biomass of all groups from the center of the cover crop (Location 4) towards the tree row (Location 1) (Table 4). In a pecan study, it was observed that cover cropped alley rows supported a higher microbial biomass compared to the tree rows (Rodriguez-Ramos et al., 2022). Several field studies showed that microbial community biomass was lower in the control treatments with bare soil compared with cover crop treatments (Buyer et al., 2010; Chavarría et al., 2016; Rankoth et al., 2019; Thapa et al., 2021). A similar positive effect of the presence of plants with high root densities on microbial biomass may explain the difference of microbial biomass between functional locations in our lemon orchard trail. Citrus trees benefit greatly from AMF associations which supports citrus trees with nutrients and help to combat negative environmental and abiotic effects on citrus health (Q.-S. Wu et al., 2017). Our data showed that both cover crops and weeds (Location 4) were capable of supporting higher AMF biomass than citrus trees growing on a berm that was kept clear of any cover crop or weeds (Location 1). Cover crops and natural grass cover were found to promote AMF root colonization of tree roots in citrus (P. Wang et al., 2016a), almond (Vasilikiotis et al., 2020), and walnut (Thioye et al., 2022) orchards. The bulk of citrus roots remains within the canopy with other roots extending outwards beyond the tree canopy into the alley row (Freeland, 2016); thus, bringing cover crops closer to the tree roots may
promote better AMF root colonization leading to better tree health. In our study, Location 2 represents the fallow area between the trees and the edge of the cover crop. Even though Location 2 was plant free, AMF NLFA biomass in that location was similar to that on the edge of the cover crop (Location 3) suggesting a potential carry over effect from the cover crop into the Location 2. Citrus trees may access Location 2 and Location 3 by extending lateral roots into alleyways and potentially receive more nutrients from AMF association. Overall, our data suggests that cereal cover crop as well as weeds can support higher microbial biomass, including AMF biomass, than tree rows, which could provide potential benefits to the trees.

3.4 Relationships among microbial groups and C cycling indicators

Arbuscular mycorrhizal fungi (AMF) are able to have synergetic interactions with saprotrophic bacteria (Cao et al., 2022; Chowdhury et al., 2022; Herman et al., 2012). AMF produces mycelia exudates leading to formation of hyphosphere that is different from the bulk soil. The AMF hyphosphere attracts various bacteria leading to a hyphosphere microbiome (L. Zhang et al., 2022). It was observed that saprotrophic fungi and bacteria can occupy and share spaces in the rhizosphere zone, while AMF and bacteria cooccurred more often in the bulk soil (Yuan et al., 2021). Based on the potential beneficial interactions between AMF and bacteria, we expected that inoculation with AMF would promote bacterial biomass over saprophytic fungi biomass. In our data set, AMF and saprophytic fungi biomass were positively correlated with most bacterial groups (Table 5) which supports previous findings on the cooccurrence of AMF or saprotrophic fungi with bacterial in soil niches. We also observed no correlation between NLFA biomass of AMF and saprotrophic fungi supporting findings that AMF and
saprotrophic fungi tend to occupy different soil niches. Contrasting to fungal NLFA, positive correlation between PLFA AMF and saprophytic fungi can be explained by potential biomarker overlap between fungi and bacteria. Certain PLFA biomarkers that are used to identify fungal biomass can be found in bacterial cells making it difficult to differentiate between microbial groups (Frostegård et al., 2011; Lekberg et al., 2022).

Soil microorganisms influence SOM formation and mineralization (Cotrufo et al., 2013; Miltner et al., 2012; Schimel & Schaeffer, 2012). We expected a positive correlation between AMF, total bacterial, and saprophytes biomass and Min C, associated with their activity in the soil. In our data set, Min C was positively correlated with all microbial groups except AMF NLFA biomass. Min C is associated with soil microorganisms’ respiration and the portion of the SOM that will be mineralized and potentially lost during microbial activities (Culman et al., 2012; Hurisso et al., 2016; Luo & Zhou, 2006) and can be increased by plant inputs that increase microbial biomass and respiration compared to bare soil (Gilmullina et al., 2023). In our data set, positive correlation between most microbial groups and Min C can be attributed to plant inputs in the cover cropped rows and weedy control rows leading to higher microbial biomass and subsequent microbial respiration and SOM mineralization. Since soil microorganisms contribute to the soil C pool through addition of microbial residues (necromass) (Khan et al., 2016; Liang, 2020; Miltner et al., 2012; Zheng et al., 2021), POXC content may reflect changes in the labile SOM pool influenced by microbial necromass contribution. Due to the potential contribution of microbial necromass to the labile carbon measured with POXC, we hypothesized that AMF, total bacterial, and saprophytes biomass would be positively correlated with POXC across treatments and locations on the orchard floor.
In addition, because multiple studies showed that AMF are linked to promotion of soil C sequestration through various mechanisms, we expected that AMF would be highly correlated with total soil C. As we hypothesized, our results showed that total soil C and POXC was positively correlated with total bacterial biomass (Table 6) suggesting potential bacterial biomass contribution to soil C via necromass. In a field study focusing on the effect of perennial grasses, corn, and soybean on soil health indicators, POXC and total soil C were found to be positively correlated with PLFA AMF, total microbial biomass, and saprotrophic fungi biomass (Pérez-Guzmán et al., 2021). In contrast to correlation between PLFA and POXC in our and other studies, NLFA AMF and saprophytic fungi biomass showed no significant correlation with total soil C and POXC. Studies that investigated effects of plant inputs, microbial live biomass, and necromass on soil C accumulation showed varied outcomes under different environmental conditions. In a conservation tillage croplands study, path analysis linking plant inputs, fungal or bacterial biomass, and fungal and bacterial necromass showed that fungi had a greater influence on SOC than bacteria (Y. Yang et al., 2022). In a legume cover crop study in orchards, partial least squares path modeling showed a causal relationship among plant inputs, microbial necromass, and SOC with fungi contributing more to microbial necromass carbon, and fungal necromass accumulated most in mineral-associated organic matter (MAOM) (Q. Hu et al., 2023). Likewise, in a study assessing proportions of microbial living biomass and non-living (necromass) contribution to SOC, it was observed that fungal necromass contributed more than bacterial necromass to SOC (Li et al., 2015). Bacterial necromass was a key contributor to soil organic carbon accumulation in temperate grasslands (X. Zhang et al., 2020) and in subtropical ecosystems during
restoration of degraded soils (Guo et al., 2021). Taking into consideration that fungal PLFA biomass can have overlap with other organisms (Frostegård et al., 2011; Lekberg et al., 2022), we considered that AMF NLFA biomass had better representation of microbial community interaction. Based on the positive correlation between AMF NLFA biomass with all microbial groups' biomass and the lack of AMF NLFA correlation with POXC, Min C and total carbon, our data suggests that AMF may influence carbon dynamics indirectly through promoting bacterial biomass.
CHAPTER 5: CONCLUSION

Intensive agricultural practices lead to negative effects on soil health and soil organic carbon content. Arbuscular mycorrhizal fungi (AMF) gained much attention in the research community due to their ability to improve plant and soil health and have a potential to promote soil C sequestration. Our study aimed to investigate the effects of inoculation with AMF and cover cropping under field conditions on soil C dynamics, microbial community structure, and the relationship between microbial community structure and carbon dynamics in a Mediterranean lemon orchard.

In contrast to our hypothesis, our results showed that inoculation of cereal cover crop with AMF had similar effect on carbon dynamics as non-inoculated cereal cover crop and weedy control treatments across the alleyways. We attributed similarities in C dynamics between treatments to potential promotion of native AMF by the non-inoculated cereal cover crop and weeds in the control treatment which was supported by PLFA and NLFA data. Thus, the impact of the cereal cover crop or weed on carbon sequestration and the AMF community overruled any potential effects of inoculation in our study. Our PLFA and NLFA results showed that three years of inoculation with a commercial AMF strain (*Rhizophagus intraradices*) did not shift the microbial community structure compared to the non-inoculated cereal cover crop. In addition, weedy control with *Malva* and felarees weeds supported microbial community in a similar way as the inoculated and non-inoculated cereal cover crop leading to no significant differences in microbial community structure between treatments. Altogether, our data showed that native AMF can be successfully promoted in the alley rows by weeds or a cereal cover crop and that AMF inoculation may not be necessary to promote
AMF in perennial citrus orchards. Since weeds are not well studied for their potential benefits in agricultural systems, our study widened the scientific community’s understanding on potential benefits of weeds in perennial agricultural systems. While other studies have found negative effects of weeds on the microbial community, we observed weeds supporting the microbial community and increasing soil organic C, similar to the benefits observed in the cereal cover crop treatment.

In our orchard study, differences in management between locations on the orchard floor impacted soil C sequestration potential in the alley rows compared to the tree rows. In the topsoil, the cereal cover crop, the weedy control, and repeated additions of tree prunings in the alley row supported more microbial biomass, including AMF, and had higher carbon sequestration potential compared to the tree row that had less plant matter input in the top soil and potentially experienced carbon loss due to pulses of C mineralization during dry-wet cycles. This suggests that carbon sequestration potential and soil health may be increased by cereal cover crop and tree pruning additions in drip irrigated lemon orchards with Mediterranean climate. In addition, orchard floor gradient assessment from the center of the cover cropped row towards the tree row with bare soil showed a potential carry over effect from the cover crop row into the fallow area between the trees and the edge of the cover crop. This suggests that potential benefits in management of alley rows can expand beyond the management zone and have a positive impacts on citrus trees.

Our data set showed that both AMF and saprophytic fungi were positively correlated with bacterial groups but not correlated among themselves supporting the fungal-bacterial cooccurrence concept where both AMF and saprotrophic fungi are able
to recruit bacteria but the two groups of fungi occupy different soil niches. Correlation assessment of linking live AMF NLFA biomass with soil C indicators showed no direct significant impact of AMF on soil C sequestration. In contrast to AMF, bacterial biomass was significantly correlated with all soil C indicators supporting studies where bacterial community was a major contributor to soil organic C accumulation. As such, our findings suggests that AMF may have an indirect influence on soil C dynamics by promoting bacterial biomass.
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