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Assessment of the Effects of Cropland Soil Remediation Strategies on the Soil Microbial Community and Tomato Agrophysiology

Evaluación de los Efectos de las Estrategias de Remediación de Suelos Agrícolas en la Comunidad Microbiana del Suelo y en la Agro-fisiología de Tomate

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TESIS DOCTORAL

Assessment of the effects of cropland soil remediation strategies on the soil microbial community and tomato agrophysiology

Evaluación de los efectos de las estrategias de remediación de suelos agrícolas en la comunidad microbiana del suelo y en la agro-fisiología de tomate

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A mis padres, mi hermana
y mi abuela

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Resumen (Spanish summary)

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La **agricultura** es una actividad económica, social y ambiental esencial para el ser humano, ya que fomenta el comercio y el empleo, y proporciona materias primas y alimentos. Desafortunadamente, muchos de los sistemas agrícolas actuales contribuyen a la degradación y reducción de la biodiversidad del suelo, al mal uso del agua y a la alteración del clima a escala mundial. Por esta razón, es necesario establecer estrategias agrícolas que permitan el desarrollo de una agricultura sostenible a largo plazo.

El agua y el suelo son dos de los pilares fundamentales de la agricultura, por lo que el manejo correcto de estos es primordial para el desarrollo de una agricultura sostenible. La escasez de **agua** es frecuente en regiones cálidas, en especial con clima árido o semiárido (como ocurre en el sureste español). No obstante, se han desarrollado técnicas de tratamiento de aguas residuales y desalinización de aguas que han incrementado el aporte de agua a los campos agrícolas. Además, se ha conseguido un mejor manejo del agua mediante la implementación de estrategias que optimizan el aporte de agua en función de las necesidades hídricas específicas de cada uno de los cultivos.

El **suelo**, por su parte, es la base de la agricultura. Está compuesto por organismos vivos, componentes orgánicos e inorgánicos, minerales, aire y agua presente en los tres estados de la materia (sólido, líquido y gaseoso). Sin embargo, el suelo es una fuente no renovable a escala humana, por lo que las prácticas agrarias deben asegurar la integridad de la calidad y salud del suelo. La evaluación de la calidad del suelo se puede realizar mediante el estudio de diversos parámetros físicos, químicos y biológicos. En concreto, los parámetros biológicos son muy sensibles a las variaciones ambientales, especialmente aquellos relacionados con los microorganismos del suelo. Estos microorganismos participan en diferentes rutas de los ciclos biogeoquímicos que tienen lugar en el suelo, teniendo papeles fundamentales en los ciclos del carbono (C), nitrógeno (N) y fósforo (P), y, por tanto, en la fertilidad del suelo. De esta manera, su estudio permite conocer

rápidamente el efecto de determinadas prácticas agrícolas sobre la calidad del suelo.

El estado de la **comunidad microbiana del suelo** se puede evaluar a partir de multitud de técnicas y aproximaciones directas e indirectas. Las técnicas directas son menos comunes debido a que solo se conocen las necesidades nutricionales y las condiciones de cultivo de alrededor del 1% de los microorganismos presentes en el suelo. En cambio, las técnicas indirectas no presentan esa limitación y permiten la evaluación de multitud de parámetros que sirven para conocer el estado de la comunidad microbiana del suelo en ese momento concreto. Existen muchos métodos, entre los que destacan la determinación de las actividades enzimáticas, la biomasa microbiana, y la composición, estructura y diversidad de la comunidad microbiana del suelo.

La actividad y biodiversidad microbiana en el suelo es esencial en la agricultura, ya que provee a las plantas de los nutrientes necesarios para su correcto desarrollo. No obstante, determinadas prácticas agrícolas provocan desajustes en el equilibrio establecido entre las comunidades microbianas del suelo. Algunas de estas prácticas están relacionadas con la aplicación de compuestos químicos (como fertilizantes y pesticidas), que pueden llegar a alterar el metabolismo de los microorganismos del suelo. En el caso concreto de los pesticidas, podemos encontrar efectos positivos, negativos o incluso neutros en función de la naturaleza de los pesticidas y de las características del suelo de estudio. Además, pueden presentar efectos específicos (afectar solo a determinadas actividades enzimáticas) o generales (cambios en la estructura y composición de la comunidad microbiana).

El uso de compuestos químicos en los campos de cultivo es una práctica muy extendida, especialmente en la agricultura intensiva. El uso de fertilizantes y de pesticidas ha permitido incrementar la producción agrícola y la calidad de los frutos. Los fertilizantes se aplican a los campos de cultivo para aportar los nutrientes necesarios a las plantas, pero este desequilibrio de nutrientes puede conducir a una pérdida de la biodiversidad del suelo. Por ejemplo, se ha visto que la aplicación de N durante largos periodos de tiempo puede producir una pérdida de diversidad en bacterias y un incremento en la susceptibilidad de las plantas a sufrir enfermedades causadas por hongos. Por ello, la aplicación de estos compuestos debe ajustarse al cultivo y suelo de estudio, pues muchos nutrientes pueden quedar retenidos en el suelo o ser lixiviados en función de las propiedades del suelo.

Los **pesticidas** son compuestos químicos o naturales que permiten controlar plagas. Se pueden clasificar de muchas formas, según el organismo diana, su estructura química, su toxicidad, su tiempo de vida media en el suelo, su modo de aplicación… Teniendo en cuenta su tiempo de vida media en el suelo, podemos encontrar pesticidas no persistentes (menos de 30 días), medianamente persistentes (entre 30 y 100 días) y persistentes (más de 100 días). El uso de pesticidas en la agricultura ha incrementado desde que se comenzaron a comercializar. Sin embargo, también han incrementado los estudios que evalúan los efectos que pueden ocasionar estos compuestos. De hecho, el uso de algunos productos se ha prohibido debido a su alta persistencia en el suelo y a los efectos nocivos generados en especies no diana, ya que pueden llegar a afectar incluso a humanos. Como consecuencia, la preocupación sobre la contaminación del suelo y la búsqueda de técnicas de remediación han aumentado en los últimos años.

Existen diversas técnicas de **remediación de suelos**. Las técnicas más utilizadas en los campos agrícolas son las técnicas *in situ*, es decir, aquellas que son llevadas a cabo en el mismo lugar. Muchas de ellas son técnicas de amplio espectro que, como la solarización y la ozonización, permiten la degradación de una gran variedad de compuestos. La **solarización** es una técnica ampliamente utilizada en el suelo para eliminar o reducir el contenido en patógenos antes del cultivo. Se suele llevar a cabo en la estación cálida del año y consiste en cubrir el suelo, previamente humedecido, con polietileno. En los últimos años, esta técnica ha obtenido resultados prometedores en la reducción de pesticidas en el suelo. Debido a que es una técnica no dirigida, puede afectar de manera general a la comunidad microbiana del suelo, llegando a comprometer la viabilidad de estos durante el posterior cultivo.

La **ozonización**, por su parte, consiste en la aplicación de ozono en forma de gas sobre el suelo. El ozono es una molécula compuesta por tres átomos de oxígeno y que presenta un alto poder oxidante. Es una técnica de amplio espectro que se ha utilizado en la descontaminación de aguas y de superficies. Recientemente, se ha evaluado su efectividad en la degradación de compuestos recalcitrantes en el suelo, obteniendo resultados alentadores. No obstante, el ozono es un gas altamente biocida, por lo que puede afectar de manera negativa a la comunidad microbiana del suelo.

No solo es importante evaluar los efectos de estas estrategias de remediación en el suelo, sino que también es primordial evaluar cómo pueden afectar dichas estrategias al **estado fisiológico del cultivo** y a la producción y calidad de los frutos obtenidos. De esta manera, teniendo en cuenta el binomio planta-suelo, se podrán establecer criterios y estrategias que se ajusten a cada uno de los cultivos para que se asegure el desarrollo de una agricultura sostenible.

La presente Tesis Doctoral se encuadra en este escenario, cuyo **Objetivo general** es evaluar cómo las diferentes estrategias de remediación aplicadas al suelo (solarización y ozonización) antes y durante el cultivo pueden alterar las características fisicoquímicas y biológicas del suelo, así como diversos parámetros fisiológicos de las plantas, y la productividad y la calidad de los frutos obtenidos. Para cumplir con este Objetivo general, esta Tesis Doctoral se ha articulado en tres capítulos en los que se han analizado los efectos de diferentes estrategias de remediación en el suelo y en la planta.

En el **Capítulo III**, se evaluaron los efectos de la solarización y la aplicación de una mezcla de pesticidas en un suelo típico de una zona semiárida mediterránea. Se analizaron los cambios provocados en las propiedades fisicoquímicas y biológicas del suelo, prestando especial atención a la comunidad microbiana del suelo. Según los resultados obtenidos en este Capítulo, la aplicación de pesticidas y el tratamiento de solarización afectaron negativamente a la comunidad microbiana del suelo. Sin embargo, los efectos más marcados fueron debidos a la solarización, la cual redujo significativamente algunas de las actividades enzimáticas evaluadas (β-glucosidasa y fosfatasa alcalina) y la biomasa microbiana, especialmente la de hongos. Por su parte, el incremento en la biomasa de Gram+ tras la aplicación de los pesticidas sugiere que el suelo de estudio contenía bacterias capaces de degradar estos compuestos.

Una vez conocidos los efectos provocados por la solarización, en el **Capítulo IV** se estudió la aplicación conjunta de solarización con ozonización, aplicada de manera superficial y en profundidad, en un suelo típico de una zona semiárida mediterránea. La ozonización ha demostrado ser eficaz en la degradación de determinados contaminantes en el suelo. Por ello, su aplicación en conjunto con la solarización puede tener un efecto sinérgico en la degradación de pesticidas presentes en el suelo. Sin embargo, ninguna de las técnicas es dirigida, por lo que se evaluaron los efectos generados sobre las características fisicoquímicas y biológicas del suelo. Además, se realizó un estudio más en profundidad de la estructura, composición y diversidad de las comunidades microbianas del suelo mediante técnicas de secuenciación de amplicones. Los resultados obtenidos en este Capítulo indican que la solarización altera las propiedades fisicoquímicas y biológicas del suelo. La aplicación conjunta de solarización y ozonización (superficial y en profundidad) no intensificó estos efectos, pero sí incrementó la degradación de los pesticidas presentes en el suelo. Además, determinadas poblaciones microbianas con capacidad para degradar pesticidas mostraron incrementos en su abundancia relativa.

Tras la evaluación de los efectos de la ozonización en el suelo, se planteó cómo afectaría la aplicación de ozono en el agua de riego durante el cultivo de *Solanum lycopersicum* L. en un suelo típico de una zona semiárida mediterránea (**Capítulo V**). Esta estrategia se suele llevar a cabo en cultivos comerciales para evitar la aparición de determinadas plagas que se transmiten por el agua y por el suelo y para incrementar la concentración de oxígeno molecular en la zona radicular de la planta. Sin embargo, el ozono es altamente oxidante, por lo que, como en el caso del Capítulo II, evaluamos las características fisicoquímicas y biológicas del suelo, además de un estudio de la estructura, composición y diversidad de las comunidades microbianas del suelo. En último lugar, se evaluó el efecto de los tratamientos sobre la agro-fisiología de la planta (contenido de nutrientes e intercambio gaseoso) y en la producción y calidad de los frutos de tomate durante el periodo de cosecha.

Los resultados del Capítulo V muestran que la aplicación de ozono en el agua de riego, comparado con la aplicación de ozono gas sobre el suelo, altera en menor medida las propiedades fisicoquímicas y biológicas del suelo. Asimismo, la diversidad, estructura y composición de las comunidades microbianas no se ven prácticamente afectadas. En cuanto a los efectos provocados en el cultivo, la aplicación continua de ozono en el agua de riego redujo significativamente la conductancia estomática. A pesar de ello, dicho tratamiento mostró una tendencia a incrementar la calidad de los frutos.

Por todo ello, se puede **concluir** que las técnicas de remediación del suelo pueden alterar las propiedades fisicoquímicas y biológicas del mismo, afectando en particular a la comunidad microbiana del suelo, un factor esencial en el mantenimiento de los ciclos biogeoquímicos y el suministro de nutrientes a las plantas. Esto se debe principalmente a su carácter de amplio espectro, ya que la solarización y la ozonización del suelo no son técnicas dirigidas. Además, es importante tener en cuenta el modo de aplicación de estas técnicas. La aplicación de ozono en forma de gas en el suelo, en combinación con solarización, parece reducir la biomasa de la comunidad microbiana del suelo, así como alterar su estructura y composición. No obstante, la aplicación de ozono en el agua de riego parece amortiguar dichos efectos.

En cuanto a los efectos de la aplicación de ozono en el agua de riego durante el cultivo de *S. lycopersicum* L., destacar que algunos parámetros agro-fisiológicos de la planta de tomate también se vieron ligeramente afectados. No obstante, el contenido en nutrientes no sufrió alteraciones debido a los tratamientos evaluados. Además, la calidad de los frutos obtenidos con la aplicación continua de ozono en el riego fue ligeramente mejor que la del resto de tratamientos.

1. Chapter I. Introduction

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1.1. Agriculture at a global scale and its importance in Spain

Agriculture is an economic, social, and environmental activity essential for human beings. Agriculture has always been closely linked to the provision of jobs, commercial activities, raw materials, and foods. However, the ever-growing population will require a 60% increase in food production by 2050 (FAO, 2020). Different crops are grown all over the world, whose cultivation depends on the geographic and climatic conditions of the region, and on the socio-economic requirements of the population (Fig. 1.1). Therefore, improved and optimized agricultural practices are required to ensure food security and food supply for the entire world population. Unfortunately, agricultural intensification has caused that many current agricultural systems contribute to land degradation, water waste, reduced biodiversity, and climate disruption on a global scale (Foley et al., 2011).

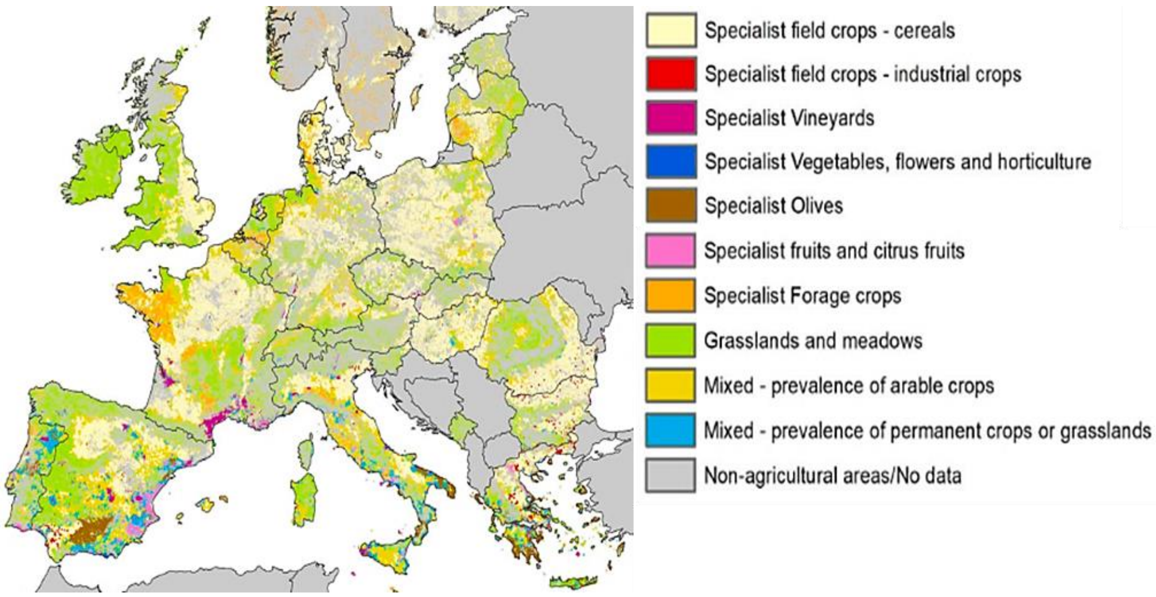

Fig. 1.1. Distribution of the 10 defined Crop Systems in Europe, based on the 50% dominance threshold. Modification of Rega et al. (2020).

It is therefore imperative to address the main problems that hinder the development of agriculture. In this regard, this Ph.D. Thesis fits with international programs and initiatives, such as Horizon 2020 program, and its societal challenges of "Bioeconomy" within "Food Security, Sustainable Agriculture and Forestry, Marine, Maritime and Inland Water Research and the Bioeconomy", as well as "Waste" within the "Climate Action, Environment, Resource Efficiency and Raw Materials". Proposal fits also very well with the "Green Deal": a new EU action plan focused to boost the efficient use of resources by moving to a clean circular economy. Besides, several projects under the Horizon Europe program aim to reduce environmental degradation, prevent the loss of soil biodiversity and improve water management by 2027.

Focusing on Spain, there is a large area of land dedicated to agriculture, reaching roughly 17 million hectares during 2019. Among the most important crops, we can find cereals, woody crops and vegetables, which account for 82% of the total land cultivated in Spain (Ministerio de Agricultura Pesca y Alimentación, 2019). However, many other crops stand out, such as legumes, tubers, industrial crops, fodder crops, citrus and non-citrus fruit trees, vineyards, olive groves, and other non-woody crops. In economic terms, Spanish agriculture grew by 4.4% in 2020 compared to the previous year (Ministerio de Agricultura y Pesca y Alimentación, 2021). However, agriculture must cope with the complex challenges posed by environmental and social changes that can limit its activity. Population growth, changes in consumer preferences, water scarcity, soil and water pollution, water quality, imbalances in nutrient cycling, and soil degradation are some of the challenges that we must address if we want to achieve a sustainable agriculture in the coming years (FAO, 2020).

Together with the social and economic changes that agriculture must deal with, climate change strongly influences agricultural practices. As abovementioned, two of the limiting factors in agriculture are related to water and soil. On the one hand, water is essential for every living organism, so its scarcity generates many challenges in all the areas in which it is involved. Mediterranean regions are highly limited by water availability for agriculture and several strategies allow increasing the water supply to agricultural fields, such as wastewater treatment and seawater desalinization (Bar-Tal et al., 2020; Bastida et al., 2018; Díaz et al., 2021; Nicolás et al., 2016; Picó et al., 2019). However, it is sometimes not enough to fulfill the agricultural requirements in water quality and quantity. Many strategies are focused on adjusting the irrigation regimes to the needs of individual crops, such as regulated deficit irrigation treatment (RDI), partial root-zone drying (PRD), and precision irrigation techniques, among others (Matteau et al., 2021; Romero-Trigueros et al., 2017; Santos et al., 2021; Wang et al., 2021a).

On the other hand, the soil is the basis on which agriculture develops, making it a fundamental component of this activity. Nonetheless, soils degrade by natural and anthropogenic factors, where stand out erosion, runoff, salinization, leaching, loss of soil biodiversity, and decline in the content of soil organic carbon and nutrients (Bastida et al., 2008; Delgado-Baquerizo et al., 2013; Lal, 2015). The development of agroecosystems depends on the maintenance of soil quality and fertility, which are determined by several indicators, including biological productivity, nutrient cycling, or physical stability and support for plant growth (Bünemann et al., 2018; Muñoz-Rojas et al., 2016; Zornoza et al., 2015).

1.2. Soil as the basis of agriculture

Soil is a heterogeneous environmental medium that provides a myriad of fundamental services to the planet, some of which include: i) **ecosystem services** such as food, water, and fiber; ii) **regulating services** that allow the regulation of the climate such as floods, disease, waste and water quality; and iii) **supporting services** that regulate the cycling of water and nutrients (Lehmann et al., 2020; Zornoza et al., 2015). Soil is considered as the natural environment in which agriculture takes place and is made up of living organisms, organic components, minerals, air, and water present in gaseous, aqueous, and solid phases (Wilpiszeski et al., 2019; Wołejko et al., 2019) (Fig. 1.2).

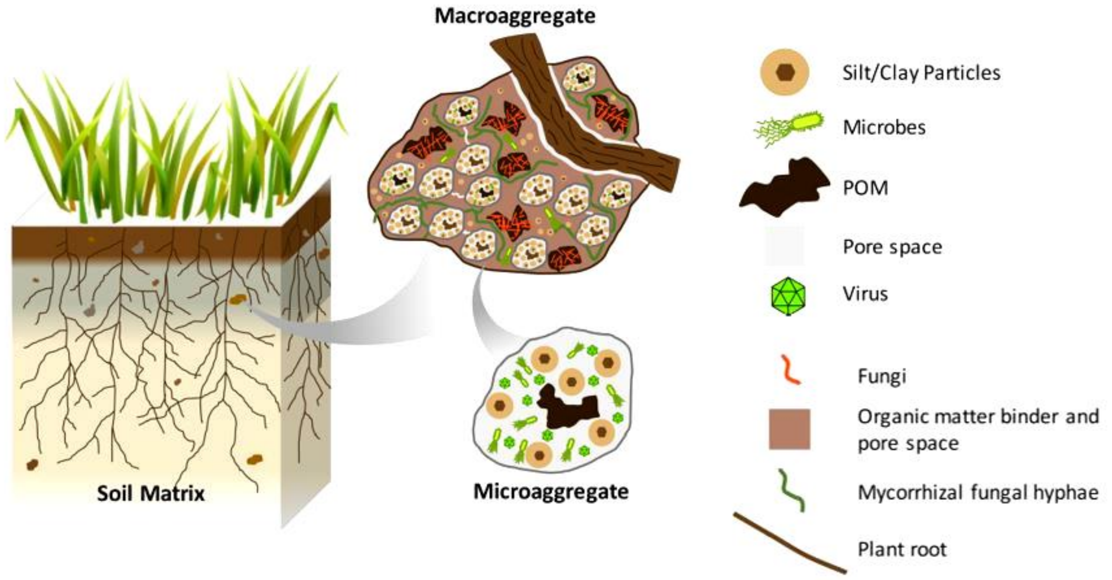

Fig. 1.2. Physical, chemical and biological components of the soil. POM, particulate organic matter. Modification of Wilpiszeski et al. (2019).

The solid phase of the soil comprises a complex structure formed by aggregates that leave spaces, called pores, filled with water or air. The presence of gaseous phase is important to provide air to all the living organisms present in the soil, from microorganisms to plant roots (Bronick & Lal, 2005; Wilpiszeski et al., 2019). Finally, the aqueous components provide water and all the soluble nutrients present in the soil (Lal, 2015). Thus, all these elements make the soil matrix dynamic and alive, always in continuous change. However, soil is a non-renewable source on a human timescale that must be carefully managed, since the overall decrease in soil quality would lead to a decrease in ecosystem services and a reduction in nature conservation (Gomiero, 2016).

The soil quality is defined as "the capacity of soil to fulfill ecological functions and provide ecosystem services to maintain biological productivity and environmental quality and enhance the plant and animal health" (Doran & Parkin, 1994; Maurya et al., 2020). In this regard, it is necessary to know which parameters define soil quality. The main indicators established as quality indexes for agricultural soils are related to a variety of physical, chemical and biological parameters (Bastida et al., 2008; Bünemann et al., 2018), among which we can highlight the following:

- Physical parameters. Water storage, texture, porosity, stability of aggregates, infiltration, and penetration resistance.
- Chemical parameters. Total organic matter, total organic carbon, pH, the content of macronutrients and micronutrients, electrical conductivity, heavy metals content, salinity, and labile C and N content.
- Biological parameters. Enzyme activities, soil respiration, microbial biomass, microbial diversity, earthworms, and N mineralization.

Maintenance of soil quality is essential in the development of the agricultural practices as it can comprise soil fertility and health. The concept of **soil health** is quite recent, and is often used as a synonym for soil quality. However, the main difference between these two concepts is the attention paid to human health: soil quality does not include the assessment of human health, whereas in soil health it is a fundamental aspect (Lehmann et al., 2020). Regardless of terminological considerations, all living and non-living organisms present in the soil contribute to the development of physical, chemical, and biological processes. Among them, the biological ones are highly sensitive to environmental disturbances, which can provide information on how the soil is affected by agricultural practices. In particular, the soil microbial community is essential in the soil fertility and sustainability. They, together with plants, are responsible of certain key processes of the biogeochemical cycles of carbon (C), nitrogen (N), and phosphorus (P) that take place in the soil matrix (Delgado-Baquerizo et al., 2013; Fierer et al., 2012).

The soil microbial community is one of the most dynamic components of the soil, as detailed above. It responds very sensitively to environmental changes, which quickly shows weather the conditions subjected to the soil are detrimental or beneficial to the soil microbial community. Knowledge of the dynamics established by the microbial groups is essential for soil functioning. Considering these characteristics, the study of small fluctuations in the soil microbial community provides insight into how agricultural practices affect the soil microbial community and whether or not it can be compromised by these practices.

The activity of soil microorganisms makes many nutrients accessible to plants, to be stored in the soil or even to be lost by leaching (Bender & van der Heijden, 2015). This will depend on the agricultural practices adopted, since some of them can cause imbalances in these cycles. The excessive use of certain compounds (such as pesticides and fertilizers) in agricultural soils could affect the soil microbial community in many ways (Lekberg et al., 2021; Soong et al., 2020). The amount of fertilizers and pesticides applied to the soil depend on the individual crop, since each one has its own nutritional requirements and associated diseases. Thus, we can find a wide range of doses in crops, from very high doses to zero doses (organic farming), depending on the agricultural practices adopted. However, only in 2019, 1.86 million tons of fertilizers (only attending to the nutrients N, P and K) and 61343 tons of pesticides were applied to the Spanish crops (FAOSTAT, 2021).

The application of fertilizers and pesticides can alter the metabolism of the soil microbial community in many different ways, which will depend on the characteristics of the compounds, the soil where they are applied, and the agricultural practices employed. In the case of fertilizers, the new nutrient conditions imposed on soil microorganisms result in modulation of their metabolism since the limiting nutrient is likely to change (Soong et al., 2020). Some studies have shown that fertilizers addition could be related to a higher incidence of plant fungal diseases, maybe because N addition could increase plant susceptibility to fungal pathogens (Veresoglou et al., 2013). Besides, long-term N application can reduce the bacterial richness and diversity, and, in contrast, favor fungal pathogens growth (Lekberg et al., 2021; Luo et al., 2019). Thus, fertilizer application should be tailored to the target crop to avoid disease occurrence due to nutritional imbalances that favor specific pathogens.

Pesticide application in agricultural fields also alters the metabolism of microorganisms present in the soil and can determine soil quality and health. Pesticides can affect soil microorganisms directly, if they are the target pest, or indirectly, if they are not the target pest but the pesticide is toxic to them. The effects of pesticides on the soil microbial community have been widely described in the literature, but they depend on the particular pesticide and soil being studied. Thus, we can find reduction in soil enzyme activities after individual exposure to acetamiprid and imidacloprid (Wang et al., 2014). In other studies, an alteration of soil microbial functionality and community composition due to chlorantraniliprole was evidenced (Wu et al., 2018). However, pesticide mixtures are not usually studied, which does not give us a realistic picture of what happens in the field when pesticides are applied as commercial mixtures.

1.2.1. Soil enzyme activities

The evaluation of the soil microbial community and its activity can be performed through a variety of techniques and approaches that may include the determination of enzymatic activities, microbial biomass, and the composition, structure and diversity of the soil microbial community.

As abovementioned, the soil microbial community plays a critical role in the maintenance of the soil quality. It is responsible of the mineralization of the organic matter, which consists in the conversion of organic compounds into inorganic ones. It is an essential part in the biogeochemical cycles that mainly are mediated by enzymes produced by microorganisms. Enzymes are proteins that catalyze the conversion of certain compounds (substrates) into other ones (products) which can have biological functions as part of plant and microbial nutrition and only microbial nutrition. We can find intracellular enzymes, whose activity develops inside the microorganisms, and extracellular enzymes, whose activity occurs outside them. Some of the extracellular enzymes are retained in the periplasm of the producing microorganisms, but most of them can be found free in the soil (Burns et al., 2013). A great number of enzymes are involved in essential soil processes, but it is impossible to evaluate all the enzymes involved. Therefore, the evaluation of some of them has been established as indicators of each one of these processes:

The **dehydrogenases** enzymes are a complex group of intracellular enzymes that are commonly used as indicators of general microbial activity. The dehydrogenase activity can increase with microbial biomass or when organic amendments are applied to the soil which provide available energy sources for the microbial community (Briceño et al., 2007). Thus, this enzyme activity is used to evaluate the overall healthy condition of the soil microbial community.

The **β-glucosidase** enzyme is an enzyme closely linked with the C cycle as it catalyzed the transformation/decomposition of organic matter in soil by the breaking of the β (1-4) glycosidic bond found in carbohydrate compounds from disaccharides to polysaccharides. Its role in the C cycle is essential because its final product is glucose, an important carbon energy source for soil microorganisms (Riah et al., 2014). As in the case of dehydrogenase, increases in this activity correlate with the application of organic amendments enriched in carbon compounds, so there is a direct relationship between the C availability in the soil and this activity.

The **urease** can be found in the soil both as intra and extracellular enzyme. It is an essential component in the N cycle as it catalyzes the hydrolysis of urea into carbon dioxide and ammonia, which is vital for plant N supply (Yang et al., 2018). The application of inorganic N sources (i.e. fertilizers) results in the reduction of this activity, which is a common practice in agricultural fields (Bowles et al., 2014).

The **acid and alkaline phosphatases** are a large group of extracellular enzymes that have activity at both low (acid phosphatase) and high (alkaline phosphatase) pH values. Phosphatases hydrolyze organic compounds releasing inorganic P, which is taken as a source of P by plants (Hayat et al., 2010). Decreases in phosphatase activity have been observed with decreases in organic matter decomposition (Delgado-Baquerizo et al., 2013).

Thus, the assessment of these enzyme activities can be a useful approach to evaluate how an agricultural management influences the activity of soil microbial communities and their involvement in nutrient cycles. However, the heterogeneous nature of the soil must be considered, since it may contain components that hinder the activity of these enzymes. In the case of intracellular enzymes, their activity is not compromised by the environment, since they are found within the microorganisms with optimal conditions for the development of their activity. In the case of extracellular enzymes, they can often experience denaturation, adsorption, inactivation and even degradation processes that can damage them. This is where mineral and humic associations become particularly significant, which provide a suitable environment for the enzymes that protects them from all the proteolysis processes (Maurya et al., 2020). Besides, the tridimensional structure of these extracellular enzymes presents confer them more stability in the soil matrix, since they present glycosylated groups or disulfide bonds (Burns et al., 2013).

1.2.2. Biomass, composition and diversity of the soil microbial community

The direct identification and characterization of soil microorganisms is extremely challenging, as only about 1% of soil microorganisms can be isolated in pure cultures (Grayston et al., 2004). Even up to date, most of the microorganisms contained in the soil have unknown nutritional requirements which makes difficult their isolation and characterization in the laboratory. Therefore, the most often utilized techniques to quantify and identify soil microorganisms are indirect ones. Many indirect techniques are available, but it is worth mentioning the quantification of microbial biomass by the analysis of fatty acid methyl esters (FAMEs), and the study of the composition and diversity of microbial communities through the identification of the operational taxonomic units (OTUs) or amplicon sequence variants (ASVs) by amplicon sequencing approaches. Although these approaches do not allow the distinction between live and dead cells, they do provide an overview of the effects of agricultural practices on the soil microbial community.

The microbial biomass can be determined by FAME content of microbial membranes. This method was firstly described by Zelles & Bai (1993) and was further modified by Schutter & Dick (2000). It consists of the esterification of fatty acids present in soil, which are assumed to come from soil microorganisms, and their subsequent determination by gas chromatography. Fatty acids are found in the cell membranes of all living organisms (Fig. 1.3), although their composition differs among different organisms. Each microbial group (bacteria, fungi, Gram positive bacteria, Gram negative bacteria or Actinobacteria) contains representative fatty acids (Frostegård et al., 2011; Montes de Oca-Vásquez et al., 2020; Vera et al., 2019). Thus, we can identify and classify them to know whether the environmental conditions studied affect the soil microbial community in a widespread way (overall reduction of microbial biomass) or in a specific one (only biomass reduction of certain groups).

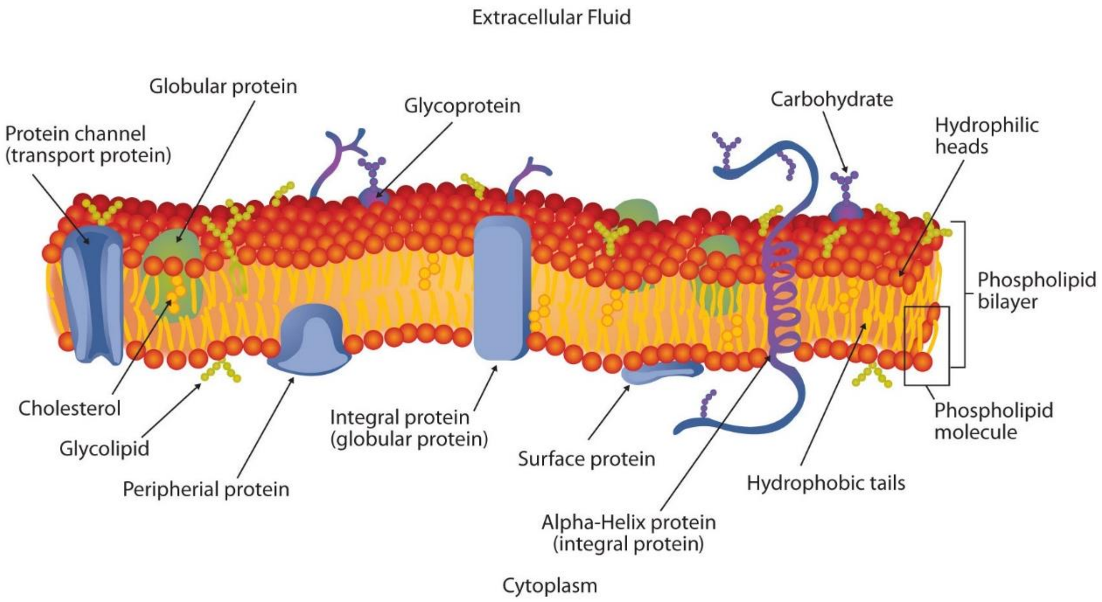

Fig. 1.3. Cytoplasmic membrane composed of a phospholipid bilayer and transmembrane proteins (Tapasya, 2014).

As a complement to the determination of microbial biomass, amplicon sequencing provides further information on the study of the soil microbial community, giving a better understanding of the fluctuations in the diversity, structure and composition of the soil microbial community due to agricultural practices. Amplicon sequencing has overcome the impossibility of studying the composition of the soil microbial community by classical techniques as laboratory cultures. It was firstly described in soil by Felske et al. (1998), but many modifications have occurred since then, as the development of technology has facilitated a breakthrough in this sort of methods.

Amplicon sequencing is a targeted approach focused on the analysis of genetic variation in specific genomic regions (Christensen et al., 2018). Currently, this technique is based on 16S and the Internal Transcribed Spacer (ITS) ribosomal RNA (rRNA) sequencing to identify bacteria and fungi from a soil sample, respectively. The prokaryotic 16S rRNA gene has nine variable regions which are frequently used for phylogenetic classification. The ITS1 region of the rRNA cistron is a commonly used DNA marker for phylogenetic of fungi classification. The results obtained from this analysis are readings of sequences that must be analyzed using bioinformatics techniques (Fig. 1.4).

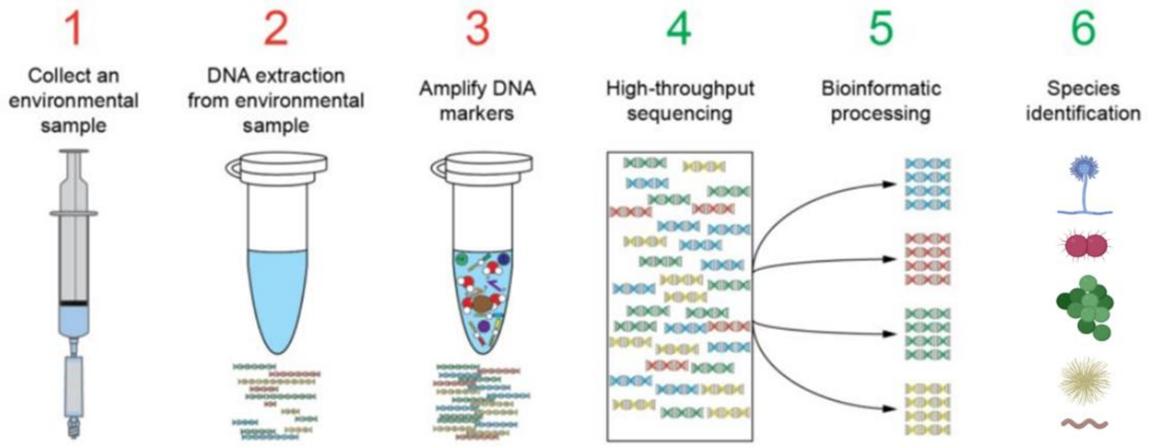

Fig. 1.4. Overview of the sequencing process from the sample collection to the bioinformatic analysis. Modification of Illumina (2021).

This field is in continuous evolution, as the available identification tools are constantly changing. Phylogenetic classification can be performed by identifying OTUs or ASVs. The difference between the two approximations is how the sequences obtained are assigned to a given phylogenetic group. The OTUs are assigned when the sequence has 97% similarity to the OTU. In contrast, the ASVs require that the sequences be 100% identical in order to be classified in a given phylogenetic group. However, both procedures are currently validated (Glassman & Martiny, 2018).

1.3. The use of pesticides and remediation techniques for pesticide-contaminated soils

Agricultural practices commonly focused on obtaining high crop yields, so plants require adequate bioavailability of nutrients and water. Improving plant nutrition and, in some cases, increasing plant resistance to abiotic and biotic stresses is achieved through the use of fertilizers and biofertilizers (Gouda et al., 2018; Kumar & Verma, 2018; Vurukonda et al., 2018). However, one of the most important biotic stresses affecting plants are pests, which generate large harvest losses that result in low crop yield. Many approaches have been developed to ensure high levels of crop productivity and high fruit quality indexes. Currently, the dominant pest control mechanism for most farmers is the application of pesticides. They are chemical or natural compounds that control pests and diseases, which can use before or during cultivation (Steingrímsdóttir et al., 2018). Depending on the target organisms, we can differentiate among rodenticides (rats and mice), insecticides (insects), herbicides (plants), fungicides (fungi), bactericides (bacteria), and larvicides (larvae) (Kim et al., 2017).

The use of these pesticides in agricultural fields has continuously been growing since they were commercialized. However, many of them can persist in agricultural soils (Fig. 1.5) in their original form or through their by-products (Silva et al., 2019). Only around 1% of the pesticides applied to the crops are estimated to reach the target pest (Sun et al., 2018), causing overuse of pesticides in agricultural fields. As a result, the remaining 99% could enter the environment causing harmful effects on non-target organisms, including humans (Arias-Estévez et al., 2008). Thus, we can find pesticides and their by-products in soil, air, and water, so the risk of pesticide contamination is widespread globally (Tang et al., 2021).

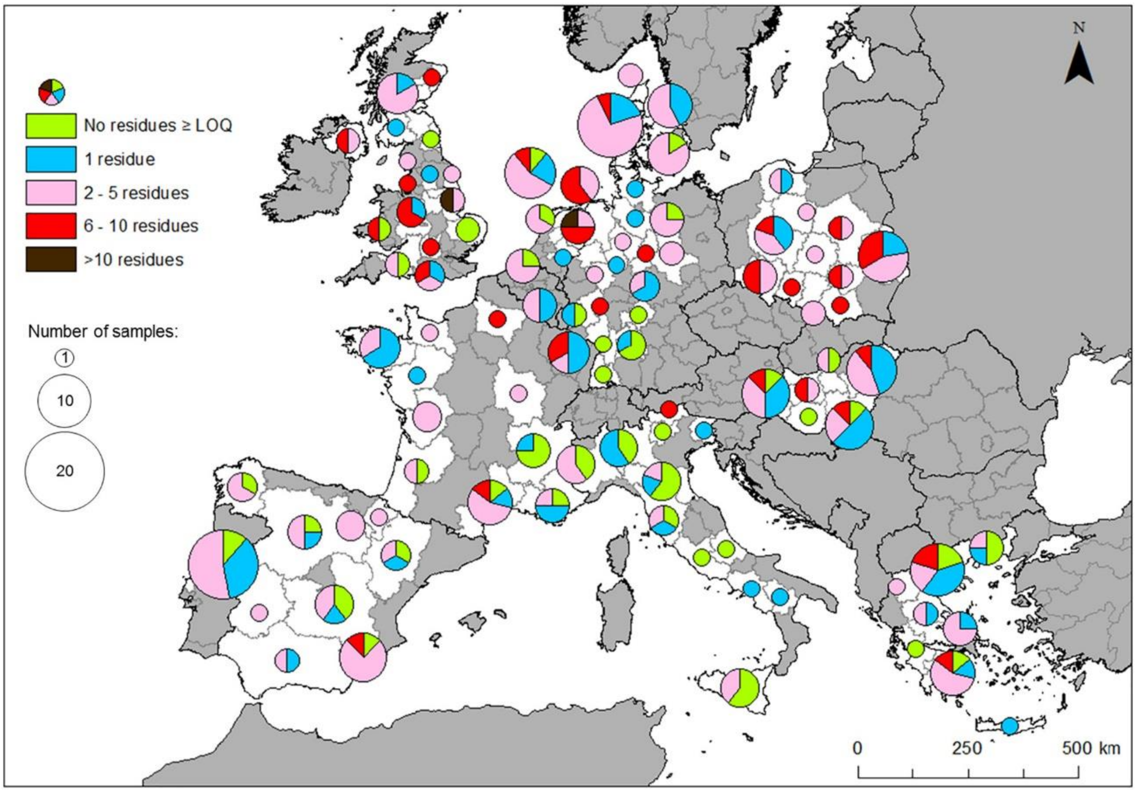

Fig. 1.5. Number of pesticide residues in EU agricultural topsoils (0–15/20 cm). The pie-charts represents the proportion of soil samples with 0, 1 and multiple pesticide residues (2–5, 6–10, >10) in each region. The size of the pie charts represents the number of samples: the larger the circle, the greater the number of samples analyzed. The white and grey areas in the map represent sampled and not-sampled regions, respectively (Silva et al., 2019).

Pesticide accumulation in soils depends on the interaction between the pesticide molecule and the soil matrix, which are determined by the physical, chemical and biological characteristics of both components (Fantke & Juraske, 2013; Wauchope et al., 2002). As an example, in the case of anionic pesticides, the soil organic matter (SOM) content determines whether the pesticide molecule retained in the soil matrix (high SOM) or the pesticide molecule is leached (low SOM) (Łozowicka et al., 2021). However, many other soil properties take part in the persistence of pesticides in soil, such as clay minerals, moisture, pH, temperature and cation-exchange capacity (Castillo Diaz et al., 2017).

Recent studies have raised awareness of the indiscriminate use of pesticides in agricultural fields. Pesticides can be classified in many ways, but if we consider the pesticide half-life, we can distinguish between non-persistent (less than 30 days), moderately persistent (30 to 100 days), and persistent pesticides (more than 100 days) (Gavrilescu, 2005). In the case of pesticides with a long half-life, the situation gets worse when they are reapplied to the crop before they have been completely degraded. This leads to their accumulation in the soil year by year, making their removal a challenge. For instance, neonicotinoids, pyrethroids, organophosphates, and organochlorines are some persistent pesticides whose accumulation in the soil could affect non-target organisms and even disperse in the environment (Sathishkumar et al., 2021; Verma et al., 2014).

Besides, prolonged exposure to pesticides could reduce soil microbial diversity by successively selecting those microorganisms able to metabolize the pesticide (Regar et al., 2019). Consequently, some of them had banned because of their accumulation in agricultural soils, their ability to damage non-target organisms, reduce soil biodiversity, and increase pest resistance (Kalia & Gosal, 2011; Kim et al., 2017; Silva et al., 2019). As an example, in 2018, the European Union banned the application of clothianidin, thiamethoxam and imidacloprid on crops pollinated by bees due to its lethal toxicity exhibited to honey bees (Declan, 2018).

Soil remediation is a current challenge to ensure sustainable agriculture in pesticide polluted sites. Many approaches have assessed this issue with varied results since pesticide molecules can establish stable complexes with soil particles, hindering their removal from the soil (Wołejko et al., 2019). Contaminated soils can be remediated using physical, chemical, and biological techniques. They can be applied: i) *in situ*, where the removal or reduction of soil pesticides content is carried out in the field; ii) *on-site*, where contaminated soil is excavated, treated in the same location and returned to the original form, and iii) *ex situ*, the soil treatment is carried out on another location (Sun et al., 2018). *In situ* restoration has become the most widely used method because of the possibility of treating larger quantities of soil. The technique chosen will depend on the soil type and the target pesticide. However, some of the approaches used with this objective are not only for pesticides remediation. In many cases, they can carry out in fields contaminated with other pollutants or even pathogens.

For instance, solarization is a well-established method that has been used for many years and is often applied in fields to control soil pathogens (Bonanomi et al., 2008; Scopa et al., 2008; Stapleton & DeVay, 1986). It consists of covering the wet soil with transparent polyethylene (Katan, 2014) to favor the increase of soil temperature. It is usually performed in the hot season when the incidence of sunlight is higher that allows reaching higher soil temperatures that increase soil disinfection (Morra et al., 2018). Recent studies have shown that increased temperature together with increased soil moisture makes pesticide molecules more easily degradable (Fenoll et al., 2017b; Vela et al., 2017). The degradation of these compounds can be mediated by chemical, physical, and/or biological processes, where degradation by the soil microbial community stands out (Kanaan et al., 2018). Therefore, solarization can be a low-cost and sustainable strategy to be used in agricultural soils to reduce or eliminate soil pathogens as well as to reduce the content of some pesticides in soils. However, because it is a non-targeted technique, it may affect other beneficial organisms with critical roles in the soil environment.

There is a wide variety of remediation techniques for contaminated soils, but most of them are not exclusive to the soil matrix. Many of them are methods already employed in water decontamination or wastewater treatment, where advanced oxidation processes stand out. These processes include a several techniques based on the generation of HO radicals, with a high oxidizing power, that facilitates the degradation of pollutants. One of these techniques is the application of ozone. Ozonation is also an emerging method widely applied in the disinfection of contaminated water and surfaces (Nagatomo et al., 2015). Ozone is a molecule composed of three oxygen atoms with a high redox potential of 2.07 V (Wang & Chen, 2020). Its use is widespread in many processes, such as drinking water disinfection, wastewater treatment, medical disinfection, and the food industry

(Remondino & Valdenassi, 2018; Rizzo et al., 2020). Besides, it has been recently studied as a remediation tool in agricultural fields due to its high reactivity with organic pollutants, as in the case of many persistent pesticides (Trellu et al., 2016). However, little information is known about the effects it can have on the soil and the crop, as it can affect several soil components besides as well as pesticides (i.e. soil organic matter, microbes etc.) due to its non-targeted nature. Therefore, it is essential to evaluate the effects that these techniques can have on the soil and the crop so that they can be applied within the framework of sustainable agriculture, currently so much promoted.

1.4. Vegetal physiology and productivity in agriculture

Soil study from an agricultural point of view is essential to implement sustainable strategies that will support the agriculture in the coming years. However, the interactions established between the soil and the plant should not be forgotten, as they are of great relevance in the development of agriculture. It is important to understand how the agricultural practices affect both plant physiology and soil fertility, in order to determine sustainable practices that can preserve the world sustainability and vegetal productivity. Adapting agricultural practices to each individual crop is a key part of agriculture because growing conditions can be very different depending on the species and variety cultivated. In general terms, a proper physiological state of plants is key to the success of agricultural crops and yield. The growing conditions and the agricultural practices must be optimized to each individual crop as its own specific nutrient and water requirements. The study of the different physiological parameters helps to adapt these plant requirements to increase their total productivity, improve their nutritional value and increase their fruit quality, as proven in various crops (celery, onion, tomato, lettuce, almond, and grapefruits, among others) (Ballester et al., 2017; Matteau et al., 2021; Nicolás et al., 2016; Romero-Trigueros et al., 2021).

1.4.1. Crop physiological assessment

Water is essential for all living beings, so one of the main parameters evaluated to know the physiological state of plants is the water status. This is particularly concerning in Mediterranean agroecosystems where water shortage can lead to the death of the plant, so the water supply must be tailored to the optimum levels of each crop. The water status of the plant can be evaluated by several methods, among which the water potential and gas exchange parameters are of great relevance. The leaf water potential shows a circadian trend throughout the day, with higher values at the beginning and end of the day and lower values at midday. Moreover, other factors can alter the leaf water potential, such as the growth phase, the age of the leaf, and the orientation and position they occupy in the plant (Bengough et al., 2011; Li et al., 2021).

In particular, gas exchange parameters are very informative and are obtained by non-destructive procedures. Among them, we highlight the importance of the net photosynthesis, stomatal conductance and the intrinsic water use efficiency (León-Sánchez et al., 2016; Linn et al., 2021). This methodology has been widely used since it was firstly described by Jones et al. (1990). Several parameters can be evaluated with this method, but net photosynthesis (A) and stomatal conductance (g_s) are of great interest to know the physiological plant condition (Berry et al., 2010).

Biotic and abiotic stresses affect plant physiology in many different ways. The net photosynthesis of leaves is one of the most sensitive physiological processes that can be reduced mainly by abiotic stress. In particular, heat stress reduce the net photosynthesis by the damage of the PSII and the reduction of the action of ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) (Zhou et al., 2017). Thus, the reduction of net photosynthesis is related to a suboptimal physiological state of the plant, which, in turn, can result in lower yields in agricultural crops. Likewise, the stomatal conductance also provides plant physiological information. Stomata are pores located on the surface of leaves that exchange gases with the atmosphere (Berry et al., 2010). The stomata are composed of two occlusive cells that allow the opening or closing of the stomata (Fig. 1.6).

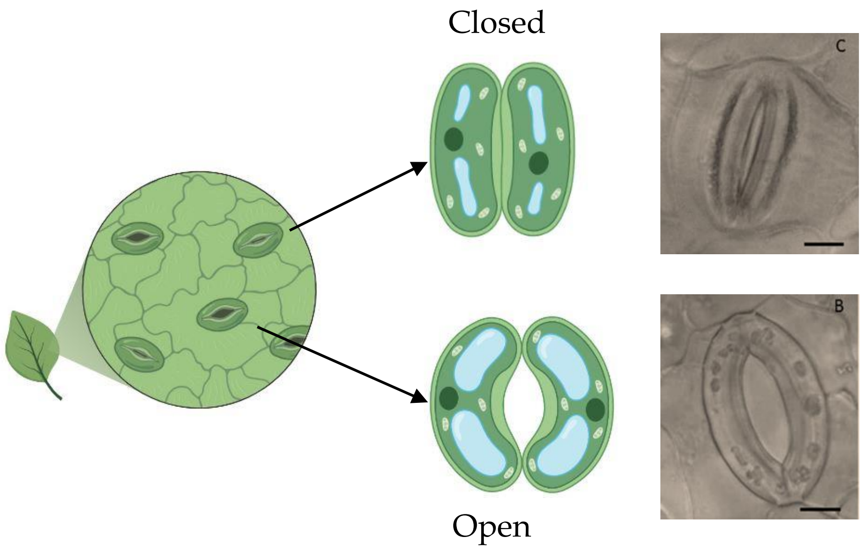

Fig. 1.6. Stomata location on the leaf and structure of the stomata in the open and closed form. Bars = 10 µm. Created with BioRender.com and a modification of Laur & Hacke (2013).

Through them, carbon dioxide necessary for photosynthesis is captured and water vapor generated by cell metabolism is released. The role of these structures in the C input and water output of the cells makes their regulation essential in the physiological balance between photosynthesis and the water status of the plant (Soong et al., 2020). Reduction in the stomatal conductance have been observed under water stress, where the plant controls water loss through closing the stomata (Dusart et al., 2019; León-Sánchez et al., 2020). Likewise, when there are oxidizing agents in the environment, such as ozone, stomata have been observed to close to protect the photosynthetic apparatus from the oxidizing action of these agents (Hu et al., 2018). Hence, the study of gas exchange parameters allows us to study in depth the effects of agricultural practices on crops. Within this PhD Thesis, the impact of ozone in water exchange and crop physiology will be deeply considered.

Together with gas exchange analyses, nutrient content also provides essential information about plant nutrition and physiology. We must distinguish between macronutrients, which are essential and found in high concentrations in plants, and micronutrients, which are also essential but required in much smaller quantities. The macronutrients are nitrogen (N), phosphorus (P), potassium (K), sulfur (S), calcium (Ca), and magnesium (Mg); and the micronutrients are boron (B), chlorine (Cl), cobalt (Co), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), and zinc (Zn). Given that plants are autotrophic the carbon source is usually obtained from atmospheric carbon dioxide. However, the remaining nutrients must be obtained from the soil, both organic and inorganic ones. At this point, microorganisms play a fundamental role, since their activity provides plants with certain nutrients essential for their growth, such as inorganic P (Philippe Hinsinger, 2011). Moreover, plants have evolved mechanisms to overcome nutrient deficiencies, such as the secretion of root exudates, that facilitate the acquisition of nutrients. Plant root exudates are made up of a complex mixture of organic acids, phytosiderophores, sugars, amino acids, inorganic ions, gaseous molecules, and enzymes that promote the acquisition of mineral nutrients necessary for plant growth (Dakora & Phillips, 2002). However, imbalances in nutrient content have been found in plants under both abiotic (heat, salinity, and drought) and biotic (pests) stress conditions (Mouhaya et al., 2010; Nicolás et al., 2016; Suzuki et al., 2014; Wahid et al., 2007; Zhou et al., 2017).

1.4.2. Tomato crop physiology, productivity, and fruit quality

Tomato (*Solanum lycopersicum* L.) is one of the most widely cultivated vegetables in the world (Fig. 1.7). The agronomic significance of tomatoes lies in their high production worldwide. In Spain, specifically, tomato production in 2019 was 5 million tons (Ministerio de Agricultura Pesca y Alimentación, 2019). Therefore, it is a suitable model plant for research studies, since it is widely cultivated and its physiology, morphology and molecular structure are well described in the literature. However, there are countless varieties of tomato that differ both in fruit size and in their sensitivity to different environmental conditions, so special attention must be given to each cultivar.

Tomato growth and development can be conditioned by different environmental and ecological factors, such as soil moisture and texture, water quality and quantity, light and climate (Ashraf et al., 2021; Kanski et al., 2021; Romero-Aranda et al., 2001; Zhou et al., 2017). The optimum relative humidity for tomato cultivation is around 60-80%, so values out of this range can affect the pollination of the flowers and the fruit, reducing its productivity. Moreover, soils with high clay content are not good for this crop, due to roots would not be able to develop properly. Their optimal growing temperatures range between 15-18 ºC at night and between 20-28ºC during the day. Temperatures below 12-15 ºC can cause problems in the development, and higher temperatures above the mentioned ranges (i.e. 30-35 ºC) cause failures in fruiting and in the development of both the plant and the root system. Fruit ripening is also influenced by environmental conditions, with alterations in fruit coloration and fruit set.

Fig. 1.7. Typical tomato plantation in the Region of Murcia.

Special attention has to be paid to the nutrient content. Most commercial crops include fertilization in their agricultural practices, which, as mentioned above, provides nutrients to plants as forms that can be assimilated by them (Liu et al., 2021). However, indiscriminate use of fertilizers can affect soil fertility, so a balance must be struck between the nutrition provided by fertilizers and that provided by soil microorganisms (Veresoglou et al., 2013). The N, P, K and Ca have a major impact on the chemical composition and physiological functions of the plant, which deficiencies can reduce crop yield by affecting the fruit (Weinert et al., 2021). As a result, maintaining optimal plant nutrition will result in high crop performance in terms of yield and fruit quality. The tomato fruit is a sink organ in which photoassimilated compounds, which are in large part sugars, accumulate. Thus, the analysis of the sugar content in tomato fruit allows us to evaluate the quality of the fruit and to know if the agricultural practices established in the crop improve or reduce the quality of the fruit (Beckles, 2012).

2. Chapter II. Scientific unit justification and objectives

Chapter II. Scientific unit justification and objectives

The aim of intensive agriculture is to ensure the highest agricultural production with the lowest possible economic losses. Crop yield in intensive agriculture is fundamental to the productive and economic efficiency of the sector. For this reason, the use of chemical compounds that maintain plant nutrition at optimum levels (i.e. fertilizers), as well as those that control pests that can damage the crop (i.e. pesticides), is common. The widespread use of these compounds, together with the persistence of some of them in soils, has led to the consideration of alternatives that generate less or no contamination in the environment. Nonetheless, it is necessary to reduce or eliminate pesticides in soils, in order to ensure a sustainable and environmental-friendly agriculture.

The sustainability of the soils depends on a multitude of physical, chemical and biological parameters. Here, the biological ones are of great importance due to their high sensitiveness to changes in the environment and because the soil microbial community is closely linked to the maintenance of the soil fertility. However, widespread adverse environmental conditions, coupled with high desired yields in agricultural crops (i.e. application of pesticides), are becoming increasingly difficult for agroecosystems to be sustainable. Agroecosystems are made up of two main components: soil and plants, and imbalances in each component and between their interactions can be detrimental. In this regard, the soil microbial community is an essential part of the soil environment since it is involved in biogeochemical cycles, and its diversity and functionality is key for buffering environmental changes in the soil.

The crop physiology and productivity are also affected by the soil fertility, as soil provide plants with nutrients, water and air required for an adequate development. Besides, there are many other factors that can affect plant physiology and, ultimately, plant yield. The main factors concern biotic and abiotic stress, but we can also find that an inadequate supply of nutrients or the application of compounds that can generate oxidative stress can negatively affect plants. Therefore, it is essential to assess how agricultural practices affect plant physiology and crop productivity. In this regard, the evaluation of the soil-plant system is also highly desirable, given the relationships between them.

This Ph.D. Thesis aims to study whether **the use of remediation approaches influences the soil microbial community in terms of functionality, structure and diversity** (Fig. 2.1). Besides, the application of these techniques before or during vegetal growing season can also alter the productivity of plants and their agro-physiological responses. Thus, the **overall objective** of this thesis is to evaluate **how different remediation techniques applied to bulk soil and soil during *Solanum lycopersicum* L. crop could alter soil chemical, biological and biochemical characteristics, as well as plant physiology, productivity and fruit quality**. To this end, three chapters are presented with three **specific objectives** addressed through them:

1) To evaluate the impact on the soil microbial community of the combination of solarization and pesticides application in a semiarid Mediterranean soil before the growing season. This objective will be assessed through the determination of some physicochemical properties of the soil, some enzyme activities associated with C, N and P cycles and the soil microbial biomass.
2) To evaluate the effects on the soil microbial community of different combinations of solarization and ozonation in a semiarid Mediterranean soil prior to the growing season. This objective will be assessed through the determination of some physicochemical properties of the soil, some enzyme activities associated with C, N and P cycles and the soil microbial biomass. Besides, the structure, diversity and composition of the soil microbial community will be determined throw the amplicon sequencing analysis.
3) To evaluate the effects on the soil microbial community and the *Solanum lycopersicum* L. crop of differential irrigation treatments with ozonated water in a semiarid Mediterranean soil. Soil parameters will include i) some

physicochemical, chemical and biochemical parameters associated with C, N and P cycles, ii) the soil microbial biomass, and iii) the structure, diversity and composition of the soil microbial community. At the plant level, we will determine physiological parameters, such as nutrient content and gas exchange parameters, and yield and fruit quality during harvest period.

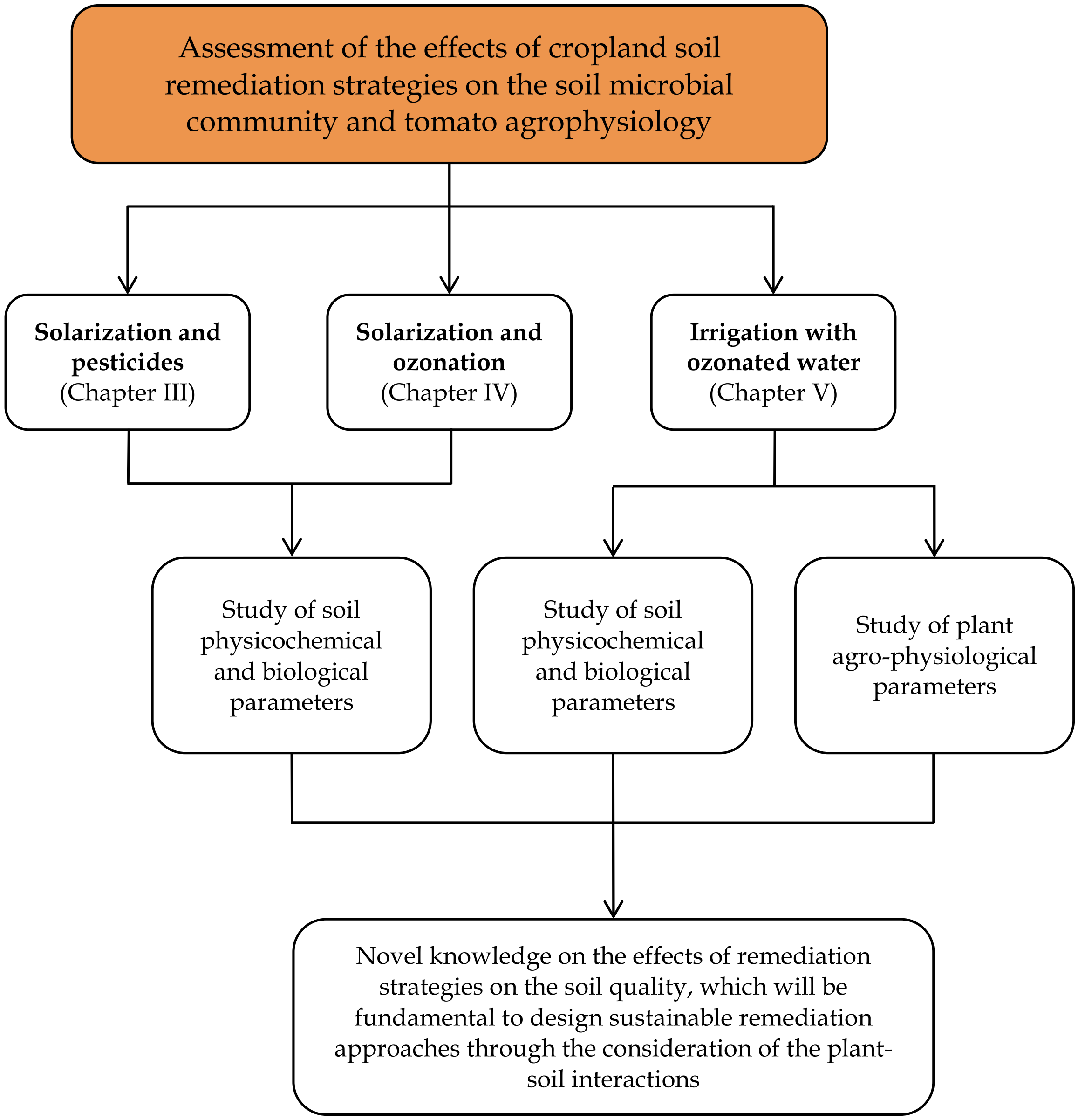

Fig. 2.1. Diagram of the purposed Ph.D. Thesis research.

3. Chapter III

Solarization-based pesticide degradation results in decreased activity and biomass of the soil microbial community

Chapter III. Solarization-based pesticide degradation results in decreased activity and biomass of the soil microbial community

Abstract

Pesticides are chemical compounds, mostly synthetic, which are used widely in agricultural fields to prevent and to control pests and soil-borne diseases. The synthetic nature of these compounds makes some of them non- biodegradable and they may accumulate in harmful concentrations in soils. Solarization seems to be a non- chemical strategy that could enhance pesticide degradation in soils. Here, we evaluate the combined impact of pesticides and solarization on the microbial community of a Mediterranean soil. For this purpose, enzyme activities, basal respiration, and the biomass and composition of the microbial community (through analysis of phospholipid fatty acids, PLFAs) were evaluated in solarized and non-solarized soils, in a 90-day greenhouse experiment with a combination of different pesticides. The degradation of the pesticides in the solarized soils was 30% greater than in non-solarized samples. However, solarization also affected the soil microbial community. The soil respiration was lowest in solarized samples without pesticides, while the enzyme activities were greater in non-solarized samples (with and without pesticides). Both the bacterial and fungal PLFA contents declined in solarized samples. The G+/G− ratio was highest in the solarized samples without pesticides and in the non-solarized samples with pesticides. Considering such impacts on the soil microbial community and the relationship of soil microbes with soil ecosystem services, the utilization of solarization must be carefully considered when adopting strategies for pesticide degradation in Mediterranean soils.

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4. Chapter IV

Combined ozonation and solarization for the removal of pesticides from soils: effects on soil microbial communities

Chapter IV. Combined ozonation and solarization for the removal of pesticides from soil: effects on soil microbial communities

Abstract

Pesticides have been used extensively in agriculture to control pests and soil-borne diseases. Most of these pesticides can persist in soil in harmful concentrations due to their intrinsic characteristics and their interactions with soil. Soil solarization has been demonstrated to enhance pesticide degradation under field conditions. Recently, ozonation has been suggested as a feasible method for reducing the pesticide load in agricultural fields. However, the effects of ozonation in the soil microbial community have not been studied so far. Here, we evaluate the combined effects of solarization and ozonation on the microbial community of a Mediterranean soil. For this purpose, soil physico-chemical characteristics and enzyme activities and the biomass (through analysis of microbial fatty acids) and diversity (through 16S rRNA and ITS amplicon sequencing) of soil microbial communities were analyzed in a 50-day greenhouse experiment. The degradation of the pesticides was increased by 20%, 28%, and 33% in solarized soil (S), solarized soil with surface ozonation (SOS), and solarized soil with deep ozonation (SOD), respectively, in comparison to control (untreated) soil. Solarization and its combination with ozonation (SOS and SOD) increased the ammonium content as well as the electrical conductivity, while enzyme activities and soil microbial biomass were negatively affected. Despite the biocidal character of ozone, several microbial populations with demonstrated pesticide-degradation capacity showed increases in their relative abundance. Overall, the combination of solarization plus ozone did not exacerbate the effects of solarization on the soil chemistry and microbial communities, but did improve pesticide degradation.

https://doi.org/10.1016/j.scitotenv.2020.143950

5. Chapter V

Assessment of ozone treatments on tomato agrophysiology and soil microbial community

Chapter V. Assessment of ozone treatments on tomato agrophysiology and soil microbial community

Abstract

Ozone has been applied in many processes (drinking water disinfection and wastewater treatment, among others) based on its high degree of effectiveness as a wide-spectrum disinfectant and its potential for the degradation of pollutants and pesticides. Nevertheless, the effects of irrigation with ozonated water on the soil microbial community and plant physiology and productivity at the field scale are largely unknown. Here, we assessed the impact of irrigation with ozonated water on the microbial community of a Mediterranean soil and on *Solanum lycopersicum* L. agro-physiology and productivity in a greenhouse experiment. For this purpose, we evaluated: i) soil physicochemical properties, soil enzyme activities, and the biomass (through analysis of microbial fatty acids) and diversity (through 16S rRNA gene and ITS2 amplicon sequencing) of the soil microbial community, and ii) the nutrient content, physiology, yield, and fruit quality of tomato plants. The effects of continuous (OZ1) and intermittent (OZ2) irrigation with ozonated water on the soil characteristics and plant physiology were distinct. The soil physicochemical and biochemical properties were slightly affected by the treatments applied. The biomasses of Gram- bacteria and fungi were decreased by OZ2 and OZ1, respectively. However, the diversity, structure, and composition of the soil microbial community were not affected by the ozone treatments. Changes in soil properties slightly affected tomato plant physiology but did not affect yield or fruit quality. Our results suggest that soil health and fertility were not compromised, but ozonated water treatments should be tailored to individual crop conditions to avoid adverse effects.

5.1. Introduction

Ozone is a molecule with a strong oxidation potential and a high degree of effectiveness as a wide-spectrum disinfectant (Mitsugi et al., 2014). In recent decades, ozone has been used in agricultural processes, drinking water disinfection, wastewater treatment, medical disinfection, and the food industry, with promising results (Remondino & Valdenassi, 2018; Rizzo et al., 2020; Wang et al., 2019b). In this regard, ozone can be applied both in the gaseous phase and dissolved in water. The application of gaseous ozone has increased, especially in the food industry, as it allows the elimination or inactivation of pathogenic microorganisms (Sengun & Kendirci, 2018). Further, the use of gaseous ozone has shown promise as a method to reduce pesticides contents in soils (Díaz-López et al., 2021; Pierpoint et al., 2003; Tamadoni & Qaderi, 2019) and as an alternative to traditional chemical pesticides for disinfection of crop fields (Nagatomo et al., 2015; Remondino & Valdenassi, 2018). However, its application in the field during the growing season could reduce plant growth and yield (Ainsworth et al., 2012; Osborne et al., 2019). Therefore, the decision about whether to use this approach should depend on the chosen crop and the application conditions (Feng et al., 2008).

In contrast, the application of ozonated water is relatively novel in agriculture and it has been used in soil-less crops to achieve substrate disinfection and increase the oxygen concentration in the nutrient solution (Graham et al., 2011b; Najarian et al., 2018; Raudales et al., 2014; Veronico et al., 2017). Ozone is unstable in aqueous solutions, so it rapidly breaks down into molecular oxygen and HO radicals (von Sonntag & von Gunten, 2015), leading to an increase in the dissolved oxygen concentration in the nutrient solution (Graham et al., 2011a). HO radicals can react with several soil organic and inorganic compounds due to their low selectivity (Rizzo et al., 2020), which could be a stress factor that influences the growth and development of plants (Zheng et al., 2020). Moreover, ozone addition to the nutrient solution could reduce soil pH, which may affect the availability of certain nutrients (Ikeura et al., 2018; Nagatomo et al., 2015). Hence, water ozonation can have positive and/or negative effects on crops depending on the specific conditions.

The high reactivity and low selectivity of ozone and HO radicals may alter the soil chemical properties with potential effects on the soil microbial community, which is fundamental for the maintenance of soil fertility and sustainability (Bastida et al., 2017; Farrell et al., 2014). One such alteration, detected in some studies, is the precipitation of metal ions, such as Fe and Mn, and micronutrient chelates (Ikeura et al., 2018; Ohashi-Kaneko et al., 2009). Moreover, ozone can rapidly react with organic matter, in the gaseous phase or dissolved in water (Ding et al., 2018; Ghahrchi & Rezaee, 2020; Rizzo et al., 2019). These reactions shift the availability of organic and inorganic nutrients, influencing plant growth and yield (Pandiselvam et al., 2019). Nevertheless, it is noteworthy that these studies were conducted on soil-less crops; there is thus a marked lack of knowledge about the effects of irrigation with ozonated water in agricultural soils. Previous studies have shown that ozone in gaseous forms influences the diversity and biomass of the soil microbial community (Chen et al., 2019), but a greater resistance of the activities of extracellular soil enzymes against harmful conditions has been found, because they are usually protected within organic matter (Burns et al., 2013; Wang et al., 2021b). However, little is known about the effects of ozone in the liquid phase on the soil microbial community, despite this being the most promising form of application.

The aim of this work was to evaluate the effects of continuous and intermittent irrigation with ozonated water on the performance of *Solanum lycopersicum* L. (tomato) and on the microbial community of a semiarid Mediterranean soil. For this purpose, we evaluated i) the soil microbial biomass, composition, and enzyme activities, together with soil chemical parameters and nutrient contents, and ii) the crop nutrient content, physiology, yield, and fruit quality. We hypothesized that continuous and intermittent irrigation with ozonated water could alter physico-chemical properties of the soil and plant agro-physiological parameters. In particular, we expected that ozone would slightly impact the soil activities of extracellular enzymes, which are usually stabilized within soil particles and organic matter (Burns et al., 2013). Given the recognized antimicrobial capacity of ozone, we expected to find a reduction in the soil microbial biomass (Mitsugi et al., 2014) and variations in the microbial community composition of the soil. Also, we expected that continuous and intermittent irrigation with ozonated water would affect plant yield and physiological parameters, as nutrient availability could be reduced due to the high oxidative potential of ozone (Ikeura et al., 2018).

5.2. Materials and methods

5.2.1. Experimental design

The experiment was conducted in a greenhouse located in Murcia, Southeastern Spain (37º46′N 0º54′W) during February-July 2020. The selected soil is a clay-loam soil (33% clay, 30% silt, 37% sand) with the following characteristics: pH (H2O) 9.03 ± 0.05; electrical conductivity (µS cm^{-1}) 136.91 ± 20.81; total organic carbon content (g kg^{-1}) 14.3 ± 1.3; total N (g kg^{-1}) 1.65 ± 0.16; alkalinity (g CaCO3 kg^{-1}) 370.91 ± 20.78. The water ozonation was performed in situ using commercial equipment provided by NOVAGRIC, S.A (utility model ES1256014). Briefly, the irrigation water was collected and filtered using several procedures: i) multi-layered sand bed filtration, ii) disc system filtration, and iii) ultrafiltration membranes filtration. Ozone was generated from atmospheric air and finally injected into the filtered irrigation water, until a redox potential of 800-850 mV was achieved.

In the greenhouse (360 m^2), the treatments were arranged in a completely randomized design with four replicates per treatment. Each replicate consisted of 39 plants of *Solanum lycopersicum* L. distributed in three rows. The plants were irrigated with drippers, with one pressure-compensated emitter per plant discharging 2 L h^{-1}, which resulted in an irrigation of 2000 m^3 ha^{-1} of water during the total growing season. All plants received the same amount of main macronutrients (N-P_2O_5-K_2O): 240-105-405, and secondary macronutrients: 44 CaO and 26 MgO (kg ha^{-1}), through the drip irrigation system. The average ambient temperature in the greenhouse during the whole trial was 23.2 ºC.

Analyses were conducted on plants and soil from the middle row of each replicate, where five plants were chosen for physiological analyses. The plants were spaced at 0.40 m within the rows and 1.00 m between the rows (2.5 plants m^{-2}). Three agronomic treatments were evaluated: 1) control treatment, 2) continuous drip irrigation with ozonated water (OZ1), and 3) drip irrigation with ozonated water, which was applied only twice: on March 5 and 27 of 2020 (OZ2). The ozone applications in the OZ2 treatment were intended to simulate soil disinfection with commercial nematicides; they were carried out one month and 1.5 months after planting. Soil samples were taken at three different times in 2020: April (T1), May (T2), and June (T3), corresponding to the pre-harvest (T1), beginning of harvest (T2), and end of harvest (T3) periods.

The soil samples were collected as follows: three soil samples, each one from the rhizosphere of a single plant, were taken at a depth of 0–15 cm and mixed to obtain one composite sample per replicate, with a total of four replicates per treatment. The samples were sieved (2 mm) and kept at 4°C for chemical analyses and at -20°C for FAME analysis and DNA extraction.

5.2.2. Soil physicochemical parameters, enzyme activities, and fatty acid methyl ester (FAME) analysis

The pH and electrical conductivity (EC) were measured in a 1:5 (w:v) aqueous soil extract, using a Crison GLP 21 pH-meter and a Crison CM 2200 conductivity-meter (Crison Hach Lange, Alella, Spain), respectively. The total N (TN), total C (TC), and total organic C (TOC) of the soil were analyzed with an Elemental Analyzer (C/N Flash EA 112 Series-Leco Truspec). The determination of the water-soluble C (WSC) and water-soluble N (WSN) of the soil was carried out using an analyzer for liquid samples (Multi N/C 3100, Analytic Jena, Germany). The ammonium of the soil was determined by a modification of the Kandeler & Gerber (1988) method. The alkaline phosphatase and β-glucosidase activities were analyzed by the method of Tabatabai & Bremner (1969) and a modification of the Eivazi & Tabatabai (1988) method, respectively. The urease activity in the soil was determined by the method described by Kandeler & Gerber (1988). The soil dehydrogenase activity was measured by a modification of the procedure of García et al. (1994).

Fatty acid methyl esters (FAMEs), hereafter fatty acids, were extracted from 3 g of soil according to Schutter & Dick (2000)and were used as indicators of the soil microbial biomass. The fatty acids i15:0, a15:0, i16:0, i17:0, 16:1ω7, cy17:0, cy19:0, 10Me16:0, and 10Me18:0 were representative of the bacterial biomass (Dungait et al., 2011; Frostegård et al., 1993), and the fatty acids 18:2ω6,9t and 18:2ω6,9c were indicators of the fungal biomass (Brant et al., 2006; Rinnan & Bååth, 2009). The Gram+ representative fatty acids were i15:0, a15:0, i16:0, i17:0, 10Me16:0, and 10Me18:0; and the Gram− representative fatty acids were 16:1ω7, cy17:0, and cy19:0 (Dungait et al., 2011; Frostegård et al., 1993). The actinobacterial representative fatty acids were 10Me16:0 and 10Me18:0 (Dungait et al., 2011). The relative abundances of all the fatty acids identified were used for the analysis of the changes in the structure of the microbial community.

5.2.3. DNA extraction and amplicon sequencing

The soil samples taken at the final sampling time were selected for examination of the diversity and composition of their bacterial and fungal communities through amplicon sequencing. The FastDNA Spin Kit for soil and the FastPrep Instrument (MP Biomedicals, Santa Ana, CA, USA) were used for the extraction of DNA from 400 mg of soil. The pair of primers 515F and 806R (Caporaso et al., 2012) was used to amplify the V4 region of the prokaryotic 16S rRNA gene and the pair gITS7 and ITS4 (Ihrmark et al., 2012) for the amplification of the fungal ITS2 region. The PCR conditions and sequencing procedure were as described in Díaz-López et al. (2021).

The sequences were processed using the USEARCH pipeline and the UPARSE-OTU algorithm (Edgar, 2013). Firstly, raw MiSeq paired-end reads from the 16S rRNA gene and the ITS2 region were assembled separately. Then, the sequences were quality-filtered, allowing a maximum e-value of 0.5 for both sets of libraries, trimmed (to 250-bp and 240-bp for the 16S and ITS2 libraries, respectively), dereplicated, and sorted by abundance (removing singleton sequences), prior chimera detection, and OTU (operational taxonomic unit) determination at 97% sequence identity. Finally, the original sequences were mapped to OTUs at the 97% identity level, obtaining two OTU tables, one for the prokaryotic community and one for fungi. The taxonomic affiliation of each OTU was obtained using the RDP (Ribosomal Database Project) taxonomic classifier (Cole et al., 2014), against 16S rRNA training set 18 for 16S rRNA gene sequences and against the UNITE Fungal ITS training set (Kõljalg et al., 2013) for ITS2 sequences, with an 80% confidence threshold in both cases. The 16S rRNA gene and ITS2 sequences were deposited in the GenBank SRA database under accession number PRJNA759554.

5.2.4. Leaf mineral analysis and gas exchange parameters

The leaf mineral content and gas exchange parameters were measured in May, at the same time as the soil was collected (T2). Middle-aged leaves (n = 10) were collected from different plants within each replicate, taking care to get a homogeneous set of leaves in terms of age and size. Then, they were washed with distilled water, dried at 60º C and ground. Leaf macronutrients (P, K, Ca, and Mg), micronutrients (Fe, Mn, and Zn), and phytotoxic elements (Na and B) were determined using an ICP-OES spectrometer (ICAP 6500 DUO; Thermo-Scientific, Waltham, MA, USA). Leaf gas exchange was measured on young, fully expanded leaves. In three plants per replicate, net photosynthesis (A) and stomatal conductance (g_s) were measured with a portable photosynthesis system (LI-6400 Li-Cor, Lincoln, NE, USA) equipped with a LI-6400/40 Leaf Chamber Fluorometer and a LICOR 6400-01 CO_2 injector. The intrinsic water use efficiency (iWUE) was determined as the A/g_s ratio. Further details are available in Nicolás et al. (2016).

5.2.5. Vegetative yield and fruit quality

Five tomato plants were selected in each replicate (a total of 20 per treatment) to evaluate the vegetative yield. Tomato fruits were sampled during the harvest period (May-July 2020). We weighed and measured the caliber of all the fruits collected in order to obtain the total yield (in kilograms) per plant. Fruit quality was assessed during the harvest period, for a total of 240 fruits per treatment (60 fruits per replicate), selected randomly. The parameters evaluated included the soluble solid content (SSC), titratable acidity (TA), and maturity index (MI). SSC and TA were determined with a handheld refractometer (ATAGO PAL-BX | ACID F5 Master Kit; Atago N1, Tokyo, Japan). Finally, MI was computed as the ratio of SSC to TA, and was used as an indication of fruit maturity at harvest, as an indication of the perception of taste by the consumer, and as an expression of juice quality.

5.2.6. Statistics

One-way ANOVA followed by the Tukey post-hoc (HSD) test was utilized to determine the significant differences ($p < 0.05$) among the treatments at the same sampling time. The normality and heteroscedasticity of the data were tested by the Shapiro-Wilk and Bartlett tests, respectively. The statistical analyses were performed with the package "stats" in the R environment (R Core Team, 2018). Principal component analysis (PCA) of the relative abundance of each fatty acid was carried out to evaluate the microbial community structure. The vectors represent the loading scores of the FAMEs. The significance of the effects of the ozonation treatments on the OTU and FAME-based microbial community structure was assessed using permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2006) with Bray-Curtis similarities and 9999 permutations. NMDS (non-metric dimensional scaling) was applied as ordination method in the case of OTU-based microbial community composition. The PCA and NMDS were performed with the PAST software, using the Bray–Curtis similarity matrix in both cases (Hammer et al., 2009). SigmaPlot (version 14.5) was used to plot all the graphs.

5.3. Results

5.3.1. Soil physicochemical properties and enzyme activities

Overall, there were no significant differences among the treatments regarding the soil chemical and physicochemical parameters, but with some exceptions.

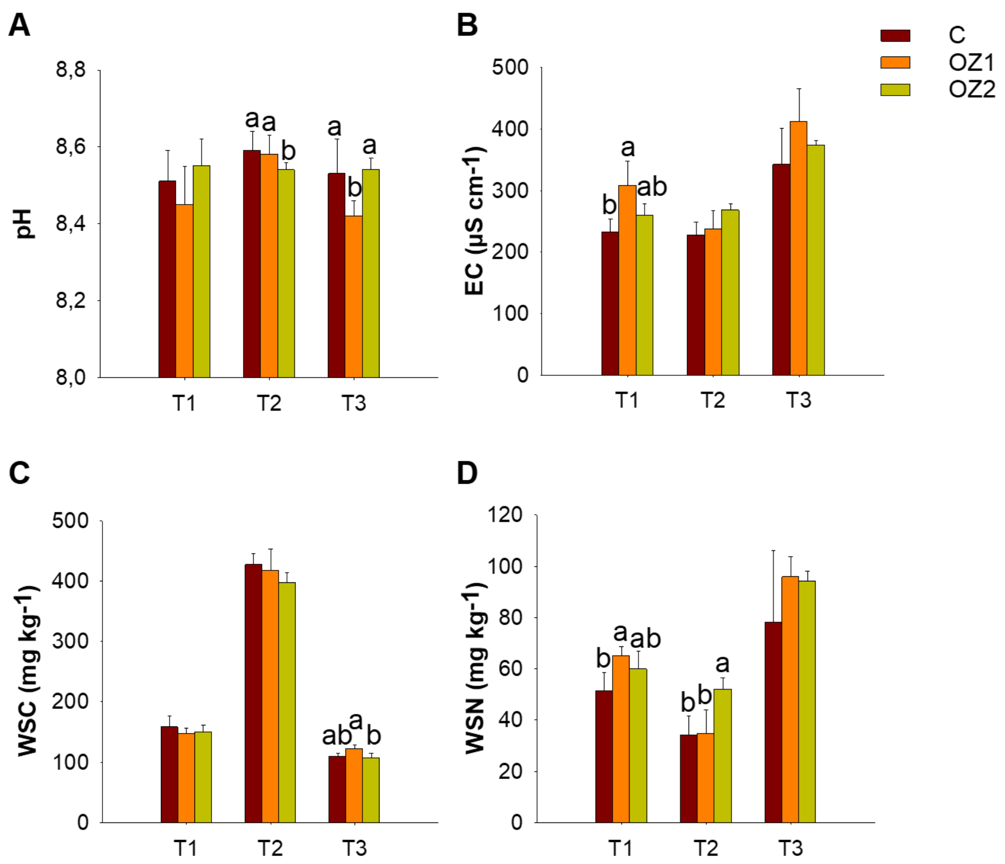

Fig. 5.1. Physicochemical and chemical properties of the studied soils: C (control), OZ1 (continuous irrigation with ozonated water), and OZ2 (intermittent irrigation with ozonated water). Time: T1 (April), T2 (May), and T3 (June). EC (electrical conductivity), WSC (water-soluble carbon), and WSN (water-soluble nitrogen). For each time point, data followed by different letters are significantly different ($p < 0.05$).

The soil pH had not undergone major changes at the end of the trial, only showing a significant (p=0.023) reduction in OZ1 with respect to the control and OZ2 (Fig 5.1A). The soil EC did not show significant differences at the end of the trial, regardless of the differences (p=0.014) among the treatments at T1 (Fig. 5.1B). The soil WSC content was significantly higher in OZ1 at the end of the trial (p=0.021), compared to OZ2 (Fig. 5.1C). Finally, the WSN content did not differ significantly among the treatments at the end of the trial (Fig. 5.1D).

At the end of the assay, there were no significant differences in the soil enzyme activities among the treatments. Nevertheless, irrigation with ozonated water did cause some slight differences in the enzyme activities, mainly at T1. Specifically, the alkaline phosphatase and β-glucosidase activities differed significantly (p=0.034 and p=0.021, respectively) among the treatments at T1 (Fig. 5.2B, C), being higher in OZ1 than in the control treatment.

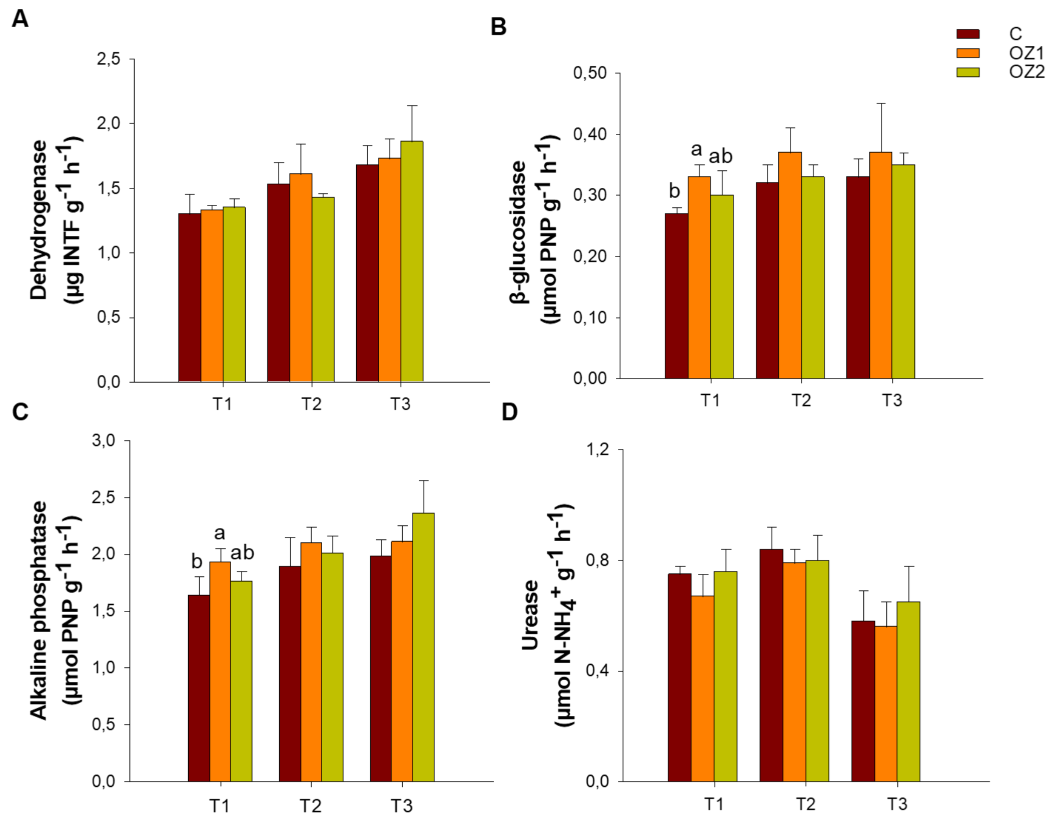

Fig. 5.2. Enzyme activities in the studied soils: C (control), OZ1 (continuous irrigation with ozonated water), and OZ2 (intermittent irrigation with ozonated water). Time: T1 (April), T2 (May), and T3 (June). For each time point, data followed by different letters are significantly different ($p < 0.05$).

5.3.2. Assessment of the soil microbial community through fatty acid analysis

Ozone had a greater effect on the bacterial fatty acid content at the beginning of the trial, while the most important differences in the fungal fatty acid content occurred at the final sampling.

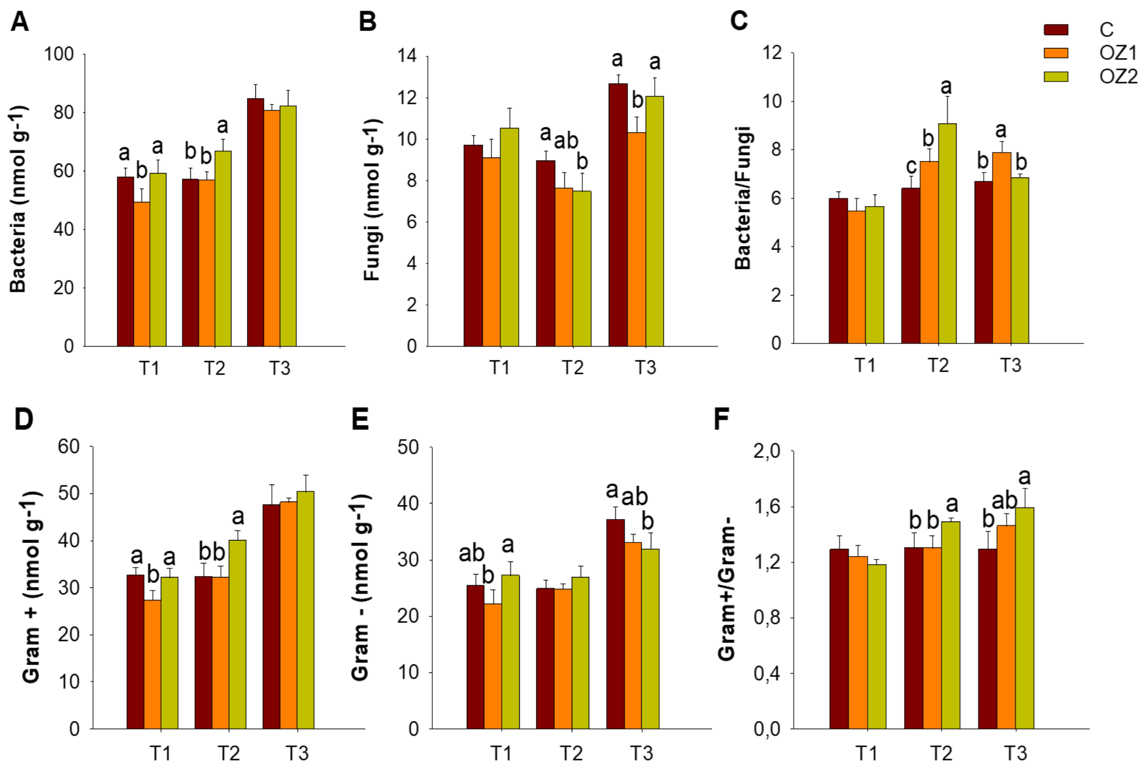

Fig. 5.3. Fatty acid contents representative of different microbial groups, and the ratios between microbial groups, in the studied soils: C (control), OZ1 (continuous irrigation with ozonated water), and OZ2 (intermittent irrigation with ozonated water). Time: T1 (April), T2 (May), and T3 (June). For each time point, data followed by different letters are significantly different (p < 0.05).

The bacterial fatty acid content (Fig. 5.3A) increased gradually in all the treatments during the assay, showing significant differences among them at T1 and T2 (p=0.015 and p=0.006, respectively). The fatty acid content of Gram+ bacteria presented a similar pattern (Fig. 5.3D). The Gram– bacterial fatty acid content (Fig. 5.3E) differed significantly among the treatments at T1 and T3 (p=0.041 and *p*=0.026, respectively). This resulted in significant differences (*p*=0.023) in the Gram+/Gram- ratio at the end of the trial, being greater in OZ2 compared to the control (Fig. 5.3F). The content of fungal fatty acids showed a response different from that of the bacterial fatty acids (Fig. 5.3B). A significant reduction in the fungal fatty acid content was observed in OZ2 (*p*=0.045) with respect to the control at T2, and in OZ1 (*p*=0.003) with respect to the rest of the treatments at T3. These shifts in the bacterial and fungal fatty acids (Fig. 5.3C) resulted in significant differences in the bacterial/fungal fatty acid content ratio at T2 and T3 (*p*=0.003 and *p*=0.002, respectively).

Besides, we analyzed the structure of the soil microbial community through PCA of the relative contents of FAMEs (Fig. 5.4).

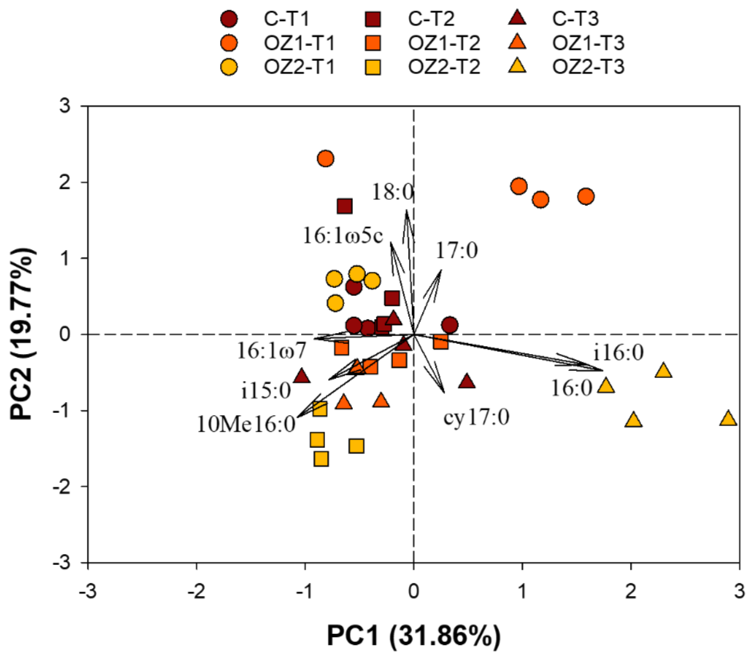

Fig. 5.4. Principal component analysis (PCA) of the fatty acids (FAMEs), showing the structure of the soil microbial communities, analyzing the three sampling times together: T1 (April), T2 (May), and T3 (June). Vectors indicate the FAMEs with a strong influence on the data distribution. C (control), OZ1 (continuous irrigation with ozonated water), and OZ2 (intermittent irrigation with ozonated water).

The sampling times were analyzed together, which provided insights into the effects of ozonated water in the soil microbial community during the whole assay.

The two-way PERMANOVA showed that there were significant differences among the treatments (F = 11.53 and p=0.0001) and among the sampling times (F = 3.91 and p=0.0001). Moreover, the sampling time x treatment interaction was also significant (F = 8. 017 and p=0.0001). PC1 explained 31.86% of the variance and PC2 19.77%. PC1 separated three groups of samples: OZ1-T1 alone, OZ2-T3 alone, and the remaining treatments. The FAMEs that received the greatest loading scores in PC1 were related to Gram+ bacteria (i15:0; i16:0), Gram− bacteria (16:1ω7), and bacteria in general (16:0). In PC2, the FAMEs that received the greatest loading scores were from Actinobacteria (10Me16:0), mycorrhizal fungi (16:1ω5c), Gram− bacteria (cy17:0), and bacteria in general (17:0; 18:0).

5.3.3. Assessment of the soil microbial community composition, structure, and diversity through amplicon sequencing

Amplicon sequencing provided a snapshot characterization of the soil bacterial and fungal communities at the end of the experiment. We found no significant differences (one-way PERMANOVA) in the structure of the prokaryotic community (F=1.089, p=0.136; Fig. S5.1A, Annex 3) nor in that of the fungal community (F=0.640, p=0.920; Fig. S5.1B, Annex 3). Similarly, there were no significant differences in the bacterial or fungal diversity (richness and Shannon index) among the treatments (data not shown).

Actinobacteria and Proteobacteria dominated the soil prokaryotic community, with a combined relative abundance of almost 60% in all the treatments (Fig. 5.5A). The composition of the soil prokaryotic community was relatively similar across the treatments. At the phylum level, we only found significant differences in the relative abundance of Actinobacteria, which was less abundant in OZ1 than in OZ2. At the order level, *Propionibacteriales* was proportionally less abundant in OZ1 than in OZ2 (Fig. 5.5B).

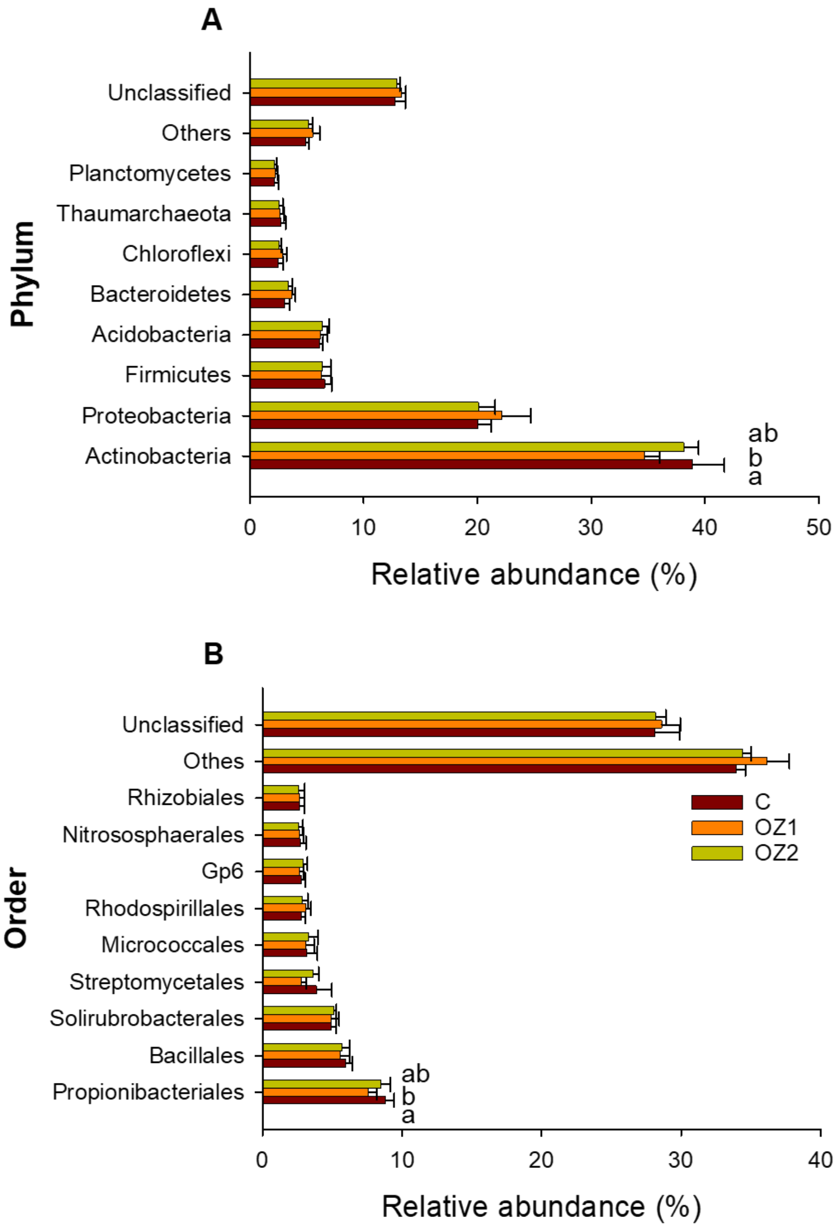

Fig. 5. Relative abundances of prokaryotic phyla (A) and orders (B) in the studied soils: C (control), OZ1 (continuous irrigation with ozonated water), and OZ2 (intermittent irrigation with ozonated water). Data followed by different letters are significantly different ($p < 0.05$).

In the case of fungi, Ascomycota was the most abundant phylum in all the treatments, having a relative abundance above 85% on average (Fig. 5.6A). The composition of the fungal communities at the order level only showed significant differences for *Eurotiales*, which was the most abundant order, followed closely by

Pezizales (Fig. 5.6B). The relative abundance of *Eurotiales* was lower in OZ1 and OZ2, in comparison with the control.

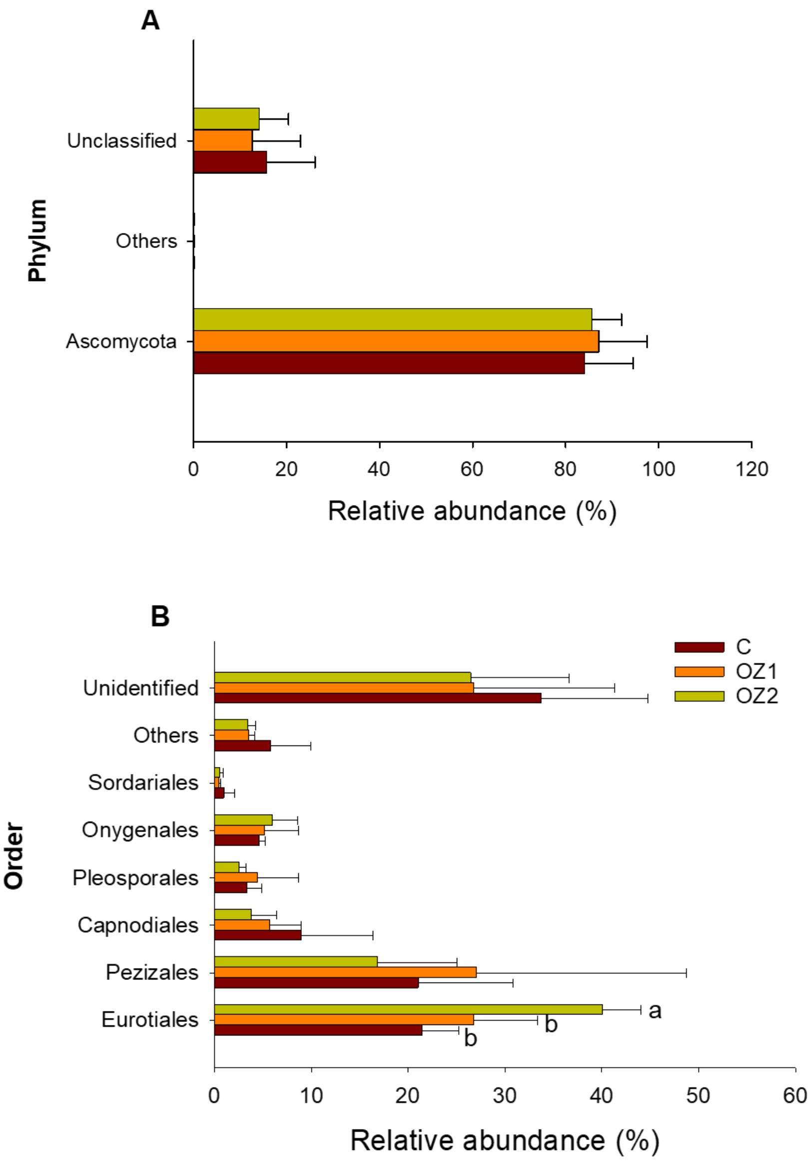

Fig. 5.6. Relative abundances of fungal phyla (A) and orders (B) in the studied soils: C (control), OZ1 (continuous irrigation with ozonated water), and OZ2 (intermittent irrigation with ozonated water). Data followed by different letters are significantly different ($p < 0.05$).

5.3.4. Leaf mineral content, gas exchange parameters, vegetative yield, and fruit quality

The response of the tomato plants was measured by analyzing the leaf nutrient content and gas exchange parameters at the beginning of the harvest period. We found no significant differences among the treatments for most of the elements detected. However, we should highlight that the contents of N, Fe, Mg, and Zn differed significantly among the treatments ($p < 0.05$). In particular, the N content was higher and the Fe content was significantly lower in OZ2 compared to the rest of the treatments, while the Mg and Zn contents were highest in OZ1 (Table 5.1). In the case of the gas exchange parameters, we observed a significant reduction in g_s and an increase in iWUE ($p < 0.01$) in OZ1 compared to the control and OZ2 treatments (Table 5.1).

Table 5.1. Leaf mineral content, gas exchange parameters, fruit productivity, and fruit quality parameters. Values represent the mean and the SD (parenthesis). C (control), OZ1 (continuous irrigation with ozonated water), and OZ2 (intermittent irrigation with ozonated water).

Mineral content	**C**	**OZ1**	**OZ2**
TN (%)	2.29 (0.09) b	2.19 (0.10) b	2.58 (0.14) a
TC (%)	37.96 (0.53)	37.51 (0.53)	37.09 (0.1.03)
Fe (mg kg^{-1})	96.40 (12.17)a	104.53 (12.57) a	71.71 (5.26) b
Mg (%)	0.57 (0.03) b	0.67 (0.06) a	0.60 (0.04) ab
Zn (mg kg^{-1})	15.06 (2.61) b	19.98 (2.37) a	16.15 (1.30) b
Gas exchange			
A (μmol CO_2 m^{-2} s^{-1})	17.98 (2.36)	18.73 (1.86)	20.18 (2.09)
g_s (mol H_2O m^{-2} s^{-1})	0.28 (0.03) a	0.22 (0.03) b	0.30 (0.05) a
iWUE (μmol CO_2 mol H_2O^{-1})	64.06 (3.99) b	85.77 (8.45) a	67.52 (7.93) b
Productivity and quality			
Productivity (kg plant^{-1})	2.05 (0.61)	1.70 (0.24)	1.97 (0.12)
TA (%)	1.01 (0.05)	0.97 (0.05)	0.95 (0.06)
SSC (° Brix)	5.27 (0.29)	5.58 (0.46)	5.10 (0.09)
MI	5.27 (0.41)	5.78 (0.36)	5.39 (0.34)

Differences in the productivity per plant were not found (Table 5.1), with the total plant productivity being 40.92, 34.03, and 39.41 kilograms for the control, OZ1, and OZ2 treatments, respectively. Moreover, the fruit quality parameters analyzed (SSC, TA, and MI) did not show significant differences among the treatments. However, it is important to highlight the trend towards higher MI values in OZ1, perhaps due to the increase in SSC in this treatment.

5.4. Discussion

5.4.1. Physicochemical and biochemical effects in soil of irrigation with ozonated water

Ozone rapidly breaks down into molecular oxygen and HO radicals in aqueous solutions (Graham et al., 2011a; von Sonntag & von Gunten, 2015). The increased amount of oxidative agents (Rizzo et al., 2020) favors reactions with the soil organic matter and the release of H^+, salts, and other compounds attached to it (Ghahrchi & Rezaee, 2020). Thus, the observed decrease in the soil pH with the continuous ozonation (OZ1) could be due to these oxidation reactions, in agreement with studies where ozone gas was applied to soil (Díaz-López et al., 2021; Mitsugi et al., 2014). Shifts in the quantity of WSC and WSN may also reflect the degradation of organic and inorganic compounds due to oxidation reactions provoked by ozone and HO radicals (Rizzo et al., 2020; Wang & Chen, 2020). Furthermore, the contents of WSC and WSN in soil also depend on the balance between root exudation and plant uptake of nutrients from the soil solution.

As widely described in the literature, plants release root exudates that contain compounds enriched in C and N - such as amino acids, organic acids, and sugars (Badri & Vivanco, 2009; Zuluaga et al., 2021). These can participate in the defense against pathogens and in nutrient solubilization and mobilization (Dakora & Phillips, 2002; Haichar et al., 2014). Significant differences in the WSC content appeared at the end of the harvest period. The lower plant yield and the increase in oxidizing agents caused by the OZ1 treatment could have resulted in the higher

WSC content in soil. On the other hand, the soil WSN content had increased at the end of the assay. Many components of WSN can be taken up by plants as a N source, mainly nitrate and ammonium (Bu et al., 2019). The accumulation of WSN in the soil could be related to the reduced nutrient uptake by tomato plants, since they were in their last stage of fruit production (Bou Jaoudé et al., 2008). The accumulation of WSN at T3 could have led to the reduction in the urease activity (Chen et al., 2015). Regarding the rest of the enzyme activities, irrigation with ozonated water had little impact. It is of note that the extracellular enzyme activities were only slightly affected. This may have been due to reactions of the oxidizing agents with other molecules before reaching the enzymes (Wang et al., 2019a) or the fact that enzymes can be protected from denaturing agents by stabilization in soil particles (i.e. clays) and stable organic matter (Burns et al., 2013).

5.4.2. Effects of irrigation with ozonated water on the biomass and composition of the soil microbial community

A reduction in biomass and a notable impact on the composition of the soil microbial community were expected, considering the antimicrobial effect of ozone (Mitsugi et al., 2014). The content of microbial fatty acids was utilized as an indicator of the microbial biomass (Fanin et al., 2019) and revealed that the bacterial biomass gradually increased during the whole trial, while the fungal biomass was increased only at the end. The increase in the resources (WSC and WSN) availability in this assay may have enhanced bacterial growth, while fungal growth is favored under low-resource conditions (Fierer et al., 2007; Strickland & Rousk, 2010; Zechmeister-Boltenstern et al., 2015). Interestingly, at the end of the assay, our results indicate that the fungal biomass was very sensitive to continuous ozonation (OZ1), while the bacterial biomass was more resistant (Bao et al., 2015), resulting in a higher bacterial/fungal biomass ratio. Previous studies have found that the fungal biomass decreases after ozonation with ozone in the gaseous phase (Díaz-López et al., 2021; Savi & Scussel, 2014). Also, we should highlight that the Gram+/Gram– bacterial ratio, a sensitive indicator of changes in the microbial community, was positively influenced by both ozone treatments. Some Gram+ bacteria are able to generate resistant structures (i.e. spores) to cope with different stress conditions (Bressuire-Isoard et al., 2018), which would explain the maintenance of the Gram+ fatty acids content at the end of our study. However, the biomass of Gram– bacteria was negatively affected by both ozone treatments, which may be due to their sensitivity to environmental perturbations (Böhme et al., 2005).

The multivariate analyses of fatty acids revealed changes in the structure of the soil microbial community across treatments and times. The community structure was influenced by treatment OZ1 at the initial sampling time (T1). These results suggest that the application of ozonated water initially affected wide microbial groups during the assay. However, these groups were able to adapt to these new conditions quickly and recover control-like values at the end of the trial, as demonstrated by the PCA. Moreover, amplicon sequencing was carried out at the end of the study to further investigate the effects generated by ozone on the composition of the soil microbial communities in greater taxonomic detail. Our results indicate that the effects of ozonated water were rather scarce. Nevertheless, there were some significant differences in the relative abundances of some taxa due to the treatments applied. The phylum Actinobacteria, including its most abundant order, *Propionibacteriales*, was negatively affected by continuous application of ozonated water (OZ1). In the case of the fungal community composition, the order *Eurotiales* was positively affected by intermittent application of ozonated water (OZ2). In the same vein, a recent study found that gaseous ozone reduced the Actinobacteria content and increased that of *Eurotiales* (Chen et al., 2019). The overall small effect of ozone on the structure and composition of the microbial community, as revealed by amplicon sequencing, may be related to the broad-spectrum effects of ozone as an antimicrobial agent (Wu et al., 2016). Nonetheless, changes in the structure and/or composition of the soil microbial communities at earlier times in the trial cannot be ruled out, especially in view of the results obtained through fatty acid analysis.

5.4.3. Plant agro-physiological effects of irrigation with ozonated water

Shifts in soil properties may affect plant physiology (Sasse et al., 2018; Zak et al., 2003). As already discussed, there were significant differences in the soil WSC and WSN contents among the treatments. Nutrient availability alterations can lead to nutrient imbalances in the plant (Lamb et al., 2011; Merino et al., 2015). The OZ2 treatment significantly increased the abundance of N and reduced that of Fe in tomato leaves, while the OZ1 treatment increased the Zn and Mg contents. These findings are encouraging since these nutrients form part of essential proteins and complexes in the plant (Hänsch & Mendel, 2009), so their deficiency can compromise the physiological balance of the plant and the fruit productivity. These results contrast with several studies showing that the use of ozonated water in hydroponic cultures can result in the precipitation of certain nutrients (Ikeura et al., 2018), making them less available to plants. Therefore, the soil matrix appears to buffer the effects of ozone on nutrient availability.

The application of ozone in irrigation water can lead to the generation of oxidative stress factors that could influence the physiological state of the plant (Savi & Scussel, 2014). Variations in the net photosynthesis (A) indicate that the growing conditions affect the plant, either positively or negatively (Masutomi et al., 2019; Zhou et al., 2017). Exposure to high concentrations of ozone gas induces the formation of reactive oxygen species that could alter the activity and content of enzymes essential to photosynthetic processes (Cailleret et al., 2018). Higher stomatal conductance has been correlated to greater damage caused by gaseous ozone (Ainsworth et al., 2012), so a reduction in stomatal conductance, as happened in our case, would be desirable to mitigate these effects (Dusart et al., 2019). However, there is little information on the effects that ozonated water could have on the photosynthetic apparatus. In our work, continuous irrigation with ozonated water decreased stomatal conductance and increased iWUE. Net photosynthesis was not affected by the presence of ozone in the irrigation water, in contrast to previous studies conducted with ozone gas (Masutomi et al., 2019; Xu et al., 2021).

Crop yield is closely related to the physiological and nutritional status of the plant. Stomatal closure has been related to lower fruit production (Nicolás et al., 2016; Romero-Trigueros et al., 2016). However, the limited effects on these parameters generated by the application of ozone in the irrigation water meant that the yields were similar in all the treatments studied. Besides, the fruit quality parameters evaluated (SSC, TA, and MI) did not show significant variations among the treatments. However, there was a trend towards higher SSC values and, in turn, higher MI values in OZ1 compared to the other treatments. The oxidative stress caused by ozone did not greatly alter the ability of the plants to produce fruit similar in quality and amount to that of the control plants, especially when ozone was applied intermittently in the irrigation water.

5.5. Conclusions

Irrigation with ozonated water affected soil properties, plant physiology, and productivity in different ways. Focusing on the soil effects, continuous irrigation with ozonated water (OZ1) had a slight impact on the soil physicochemical and biochemical properties, while intermittent irrigation with ozonated water (OZ2) did not alter these properties. At the end of the assay, there was a reduction in the fungal biomass with OZ1 and in the biomass of Gram- bacteria with OZ2. However, the diversity, structure, and composition of the soil microbial community were not affected by the ozone treatments. Our results suggest that soil health and fertility were not compromised, despite the broad-spectrum antimicrobial properties of ozone. Ozonated water slightly affected the physiology of the tomato plants. The stomatal conductance was decreased by the OZ1 treatment, but with no effect on yield or fruit quality. Therefore, ozonated water treatment should be adjusted according to the plant species and conditions involved, to avoid adverse effects on the crop and to maintain soil health and fertility for sustainable agricultural management.

6. Chapter VI. General discussion

Chapter VI. General discussion

Intensive agriculture has widespread the use of pesticides all over the world in order to ensure food production. Pesticides are usually applied to the crop fields to control soil borne diseases caused by bacteria, fungi, insects, nematodes, and, weeds, among others. However, pesticides have been found in all types of soils around the world (Silva et al., 2019). Thus, concerns have been raised in recent years because pesticides can provoke harmful effects on non-target organisms and detrimental effects in ecosystems. They can also contaminate other natural sources - such as groundwater and air - or even accumulate in the food chain, which could ultimately affect human health (Bento et al., 2016; de Souza et al., 2020; Doolotkeldieva et al., 2018; Goulson, 2013).

In this PhD Thesis, different soil remediation techniques have been evaluated that, alone or in combination, have been shown to increase the degradation of long half-life pesticides in the soil (Chapters III and IV). Furthermore, the effect of ozone application in irrigation water has also been evaluated, which is a technique widely used in the field as it allows sanitizing irrigation water in a preventive way to possible water-borne or soil-borne pests (Chapter V).

As discussed in the preceding chapters, the different techniques for remediation of soils contaminated with pesticides can alter the physicochemical, biochemical and biological characteristics of the soils. Solarization is a widely used method to disinfect soils prior to cultivation in order to reduce or eliminate soil-borne pathogens. However, it can also be employed in the reduction of pesticides in soils, since the increase in temperature and moisture favors the degradation of these compounds (Fenoll et al., 2010b; Vela et al., 2017). The increased temperature and moisture can also favor the activity of certain microorganisms that can use these molecules as an energy source. As detailed in the Chapter III, solarization increased the degradation of pesticides, but in turn, the microbial biomass was negatively affected, as expected due to its non-targeted nature (Kanaan et al., 2018).

Thus, the structure, composition and functionality of the soil microbial community were slightly affected by solarization.

We observed a similar pattern with the combination of ozonation (superficial and in depth) and solarization. Ozone results in a synergistic effect with solarization, so the degradation of pesticides was increased compared to solarization alone. Ozone preferentially reacts with soil organic matter, so the physicochemical properties of the soil were significantly altered. As a result, changes in pH, EC, WSC, and WSN (especially NH_4^+) have been observed with the application of ozone in gaseous form on soil without cultivation and with the application of ozone in the irrigation water during cultivation (Chapters IV and V).

The soil microbial community was affected by the different combinations of solarization and gaseous ozonation, but it is noteworthy that ozone did not enhanced the solarization effects. However, some significant differences were observed between solarization with surface ozonation (SOS) and solarization with deep ozonation (SOD) on the soil microbial biomass, attending to overall bacterial and fungal communities. The SOD treatment affected the fungal community to a greater extent compared to SOS treatment. In contrast, the bacterial community was more affected by the SOS treatment than the SOD one. The soil microbial functionality was also altered, mainly by the undergone changes in soil physicochemical and biochemical properties. The degradation of carbon-rich compounds increased the water-soluble C content in SOS treatment, which reduced the activity of β-glucosidase. The increase in water-soluble N content, mainly NH_4^+, also affected to the urease activity, which was drastically reduced in all cases studied.

The shifts observed in the soil characteristics in Chapter IV were consistent in Chapter V. However, the slight effect of irrigation with ozonated water on soil physicochemical and biochemical characteristics is remarkable. Significant differences were found only in microbial biomass content and soil microbial community structure when ozone was application with irrigation water. These results highlight the susceptibility of the soil microbial community to small changes in the soil, practically undetectable by considering other parameters.

The assessment of ozone application through irrigation water also revealed that water appears to buffer the effect of ozone, which could be due to its rapid dissociation based on its low water solubility (von Sonntag & von Gunten, 2015; Wang & Chen, 2020). However, the oxidizing power of ozone is not completely wasted as highly reactive protons are released when ozone dissociates in water. The high content of oxidizing molecules in the rhizosphere environment could alter plant physiology, as described in Chapter V. The continuous application of ozone in irrigation water (OZ1) causes lower stomatal conductance compared to the control, with a consequent increase in iWUE. However, these better physiological conditions do not necessarily lead to a better crop yield, since it is in this treatment where we found the lowest yield rate. On the other hand, this slight reduction in yield could have improved the quality of tomato fruits produced by OZ1-treated plants. The observed increase in SSC in OZ1 compared to the control indicates that there is a greater accumulation of carbon compounds (mainly sugars) in the fruits.

7. Chapter VII. Conclusions

Chapter VII. Conclusions

The **overall conclusions** derived of this PhD Thesis is that soil remediation techniques can alter soil physicochemical, chemical and biological properties, taking special attention on the soil microbial community, which is essential for the maintenance of the biogeochemical cycles and nutrient supply to plants. This is mainly due to their broad-spectrum character as soil solarization and ozonation are not targeted techniques. Besides, some agro-physiological parameters of the tomato plant were also slightly affected. Nevertheless, the quality of the fruits obtained with the continuous irrigation with ozonated water was slightly better than the other treatments.

Thus, the **specific conclusions** obtained in this PhD Thesis are the following:

1) Soil solarization impacted negatively the activity and the biomass of the soil microbial community, and changed community structure.
2) Soil solarization can change the physicochemical and chemical characteristics of the soil.
3) The combination of solarization plus ozone - with some exceptions - did not exacerbate the effects of solarization on the soil chemistry and microbial communities, but did improve pesticide degradation. However, several microbial populations with known pesticide-degradation capacity were able to resist soil ozonation and their relative abundances increased.
4) Superficial ozonation (SOS) produced higher NH_4^+ concentrations and soil electrical conductivity values than deep ozonation (SOD) and affected the activity of some enzymes with a key role in soil fertility.
5) Continuous irrigation with ozonated water (OZ1) had a slight impact on the soil physicochemical and biochemical properties, while intermittent irrigation with ozonated water (OZ2) did not alter these properties.
6) The diversity, structure and composition of the soil microbial community were not affected by the ozone treatments, although at the end of the trial

there was a reduction in the biomass of fungi with OZ1 and in the biomass of Gram negative bacteria with OZ2.

7) The physiological parameters of the tomato plants were slightly affected by ozone treatments. Only the stomatal conductance was decreased by the OZ1 treatment. However, no significant variation in crop yield or fruit quality was observed.

8. Chapter VIII. References

Chapter VIII. References

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9. Chapter IX. Annexes

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9.1. Annex 3. Supporting information of Chapter V (Assessment of ozone treatments on tomato agrophysiology and soil microbial community)

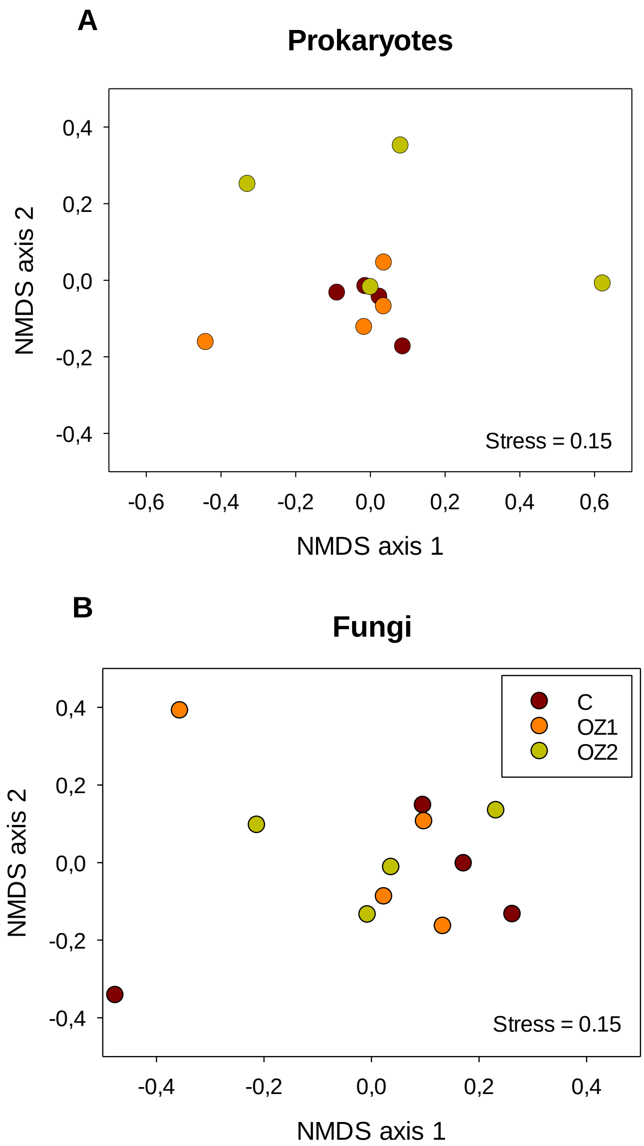

Figure S5.1. Non-metric multidimensional scaling (NMDS) ordinations based on the Bray–Curtis similarity of the OTU-based prokaryotic (A) and fungal (B) community structures (2742 OTUs for prokaryotes and 514 for fungi). C (control), OZ1 (continuous irrigation with ozonated water), and OZ2 (intermittent irrigation with ozonated water).

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