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Study of ecological hydromulching as a sustainable alternative to plastic films in horticultural crop production: effects on soil quality, plant growth and physiology

Estudio del hidroacolchado ecológico como alternativa sostenible al acolchado plástico en horticultura: efectos sobre la calidad del suelo, el crecimiento y la fisiología de la planta

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Achievements

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> "How lucky I am to have something that makes
> saying goodbye so hard." (Winnie-the-Pooh).

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Table of contents

Abbreviations and symbols

ABA	Abscisic acid
ACC	1-amino-cyclopropanecarboxylic acid
A_{CO2}	Net CO_2 fixation rate
AMF	Arbuscular mycorrhizal fungi
APX	Ascorbate peroxidase
BS	Bare soil
C	Non-covered substrate
CAT	Catalase
C:N	Carbon to nitrogen ratio
CEC	Cation exchange capacity
CKs	Cytokinins
cwInv	Cell-wall bound invertase
cytInv	Cytoplasmic invertase
DAT	Days after transplanting
DW	Dry weight
E	Transpiration rate
EC	Electrical conductivity
ERM	Extraradical mycelium
ETc	Crop evapotranspiration
Fv/Fm	Maximum quantum efficiency rate
FW	Fresh weight
GAs	Gibberellins
gs	Stomatal conductance
IAA	Indole-3-acetic acid
IRM	Intraradical mycelium
iP	Isopentenyladenine
JA	Jasmonic acid
LA	Leaf area

LLDPE	Linear low-density polyethylene
LS	Leaf succulence
LWC	Leaf water content
MS	Mushroom substrate-based hydromulch
NUE	Nutrient use efficiency
PCA	Principal components analysis
PE	Polyethylene film
PSII	Photosystem II
RH	Rice hulls-based hydromulch
ROS	Reactive oxygen species
RWC	Relative water content
SA	Salicylic acid
SN	Total soil nitrogen
SOC	Soil organic carbon
SOD	Superoxide dismutase
SOM	Soil organic matter
SR	Soil respiration
TW	Turgid weight
tZ	Trans-zeatin
vacInv	Vacuolar invertase
WS	Wheat straw-based hy-dromulch
WUEi	Intrinsic water-use efficiency
ZR	Zeatin riboside
Ψs	Osmotic potential

Spanish summary

Uno de los problemas más importantes que limitan el desarrollo del sector agrícola en la cuenca mediterránea es la escasez de recursos hídricos como consecuencia de su escasa pluviometría. El cambio climático afecta los recursos hídricos, reduciendo la recarga de aguas subterráneas y la calidad del agua, aumentando los conflictos entre usuarios, la degradación de los ecosistemas y la salinización de los recursos hídricos. El Grupo Intergubernamental de Expertos sobre el Cambio Climático (IPCC) afirma en su último informe que el cambio climático está agravando los fenómenos extremos haciéndolos más frecuentes e intensos, impactando intensamente en las actividades agrícolas. Se prevé que la exposición al déficit hídrico aumente y que, junto con la baja disponibilidad de nutrientes en el suelo, el crecimiento y el desarrollo de las plantas se verán considerablemente afectados, provocando una reducción de los rendimientos de los cultivos y grandes pérdidas económicas. En condiciones de estrés hídrico el crecimiento de las plantas, así como su capacidad fotosintética se ven reducidos, inhibiendo la actividad de importantes enzimas metabólicas, cambiando la composición de los metabolitos sintetizados y acumulados y afectando a la estabilidad de diversas proteínas, lo que provoca un estado de desequilibrio metabólico general. Para mejorar la supervivencia en estas condiciones, las plantas adoptan un conjunto de ajustes fisiológicos mediados principalmente por fitohormonas, mediante las cuales se regulan gran cantidad de procesos celulares en las plantas superiores, y se coordinan diversas vías de transducción de señales durante la respuesta al estrés abiótico.

La alcachofa (*Cynara candunculus* var. *scolymus* L.) y la escarola (*Cichorium endivia* L.) son dos especies de cultivo pertenecientes a la familia Asteraceae de gran importancia en el área mediterránea. Gran parte de la producción hortícola en esta zona se lleva a cabo bajo acolchado, lo que ha ayudado a mejorar la productividad de los cultivos debido a su efecto sobre la retención de agua. Las consecuencias del uso de acolchados se traducen en una producción más temprana, por el efecto amortiguador de las variaciones de temperatura, un mejor control de la población de flora arvense y un importante ahorro de agua de riego. De entre los materiales de acolchado utilizados, destaca el uso del polietileno lineal de baja intensidad, debido a su bajo coste y fácil instalación. La intensificación en el uso de materiales plásticos en la agricultura durante muchas décadas, si bien ha incrementado significativamente la productividad, ha provocado también crecientes efectos nocivos sobre el agroecosistema. Se han desarrollado diferentes alternativas para minimizar el impacto ambiental del acolchado plástico. Uno de los enfoques más interesantes, desde un punto de vista medioambiental, para reducir el uso de materiales plásticos es el uso de un material innovador y sostenible como el hidroacolchado. Los hidroacolchados, o acolchados líquidos, consisten en una mezcla semilíquida que se produce a partir de la combinación de agua con un polímero de tipo lignocelulósico, junto con residuos vegetales procedentes de cultivos, que posteriormente solidifica en el suelo, simulando el efecto de los acolchados tradicionales. Cuando el material se deshidrata, se endurece y ejerce su acción de acolchado, siendo un material biodegradable y económico. Los diferentes hidroacolchados estudiados en esta tesis se han desarrollado mezclando varios componentes, como pasta de papel reutilizado, y residuos de diferentes cultivos: paja de trigo, cascarilla de arroz y sustrato que ha sido utilizado previamente en cultivo de champiñón. El uso de hidroacolchados, como un tipo de acolchado orgánico, puede resultar en una mayor eficiencia en el uso del agua al evitar la evaporación del suelo, y aumentar la capacidad de retención de agua del suelo y la disponibilidad de nutrientes debido a la descomposición del abono orgánico, entre otros.

Algunos autores han reportado que el acolchado plástico y otros tipos de acolchado orgánico como la paja de trigo producen una respuesta positiva en cultivos hortícolas al mejorar los parámetros de calidad del suelo y aumentar el rendimiento de los cultivos. Otros estudios han afirmado que el uso de acolchados orgánicos en especies hortícolas mejoraba la calidad física del fruto y su contenido de agua, así como el rendimiento comercial. Hasta donde sabemos, el conocimiento del efecto del hidroacolchado en las características del suelo, que influyen en el rendimiento y la calidad del cultivo, es muy escaso. Por ello, en un primer experimento, se realizó un estudio que tuvo como objetivo evaluar el uso de hidroacolchados, en las propiedades fisicoquímicas y biológicas del suelo y su influencia en las características de rendimiento y calidad en un cultivo de ciclo largo como es la alcachofa, en comparación con el acolchado plástico y el suelo sin acolchado. Las plantas de alcachofa (*Cynara candunculus* var. *scolymus* L.) cv. "Symphony", se cultivaron en condiciones reales de campo utilizando tres tratamientos de hidroacolchado en función de su materia prima vegetal: cascarilla de arroz (RH), paja de trigo (WS) y sustrato postcultivo de champiñón (MS); y dos tratamientos control: suelo cubierto con polietileno lineal de baja densidad (PE) y suelo desnudo (BS). Para cada tratamiento se midieron los parámetros relacionados con la calidad del suelo: humedad, conductividad eléctrica, contenido mineral, materia orgánica, carbono orgánico, capacidad de intercambio catiónico y respiración de suelo; y las propiedades físicas y químicas del fruto de alcachofa: color, firmeza, peso y diámetro de fruto, contenido mineral, proteína y carbohidratos. La materia orgánica y el carbono orgánico del suelo aumentaron en todos los tratamientos de hidroacolchado, especialmente con la cobertura RH. Los suelos hidroacolchados mostraron una mayor humedad del suelo, especialmente en el suelo cubierto con WS, y una mejora de los elementos K^+, P, Cu^{2+}, Mn^{2+} y Zn^{2+}, particularmente en el suelo cubierto con MS. Los tratamientos con acolchado produjeron el mayor rendimiento del cultivo, mientras que el menor rendimiento se obtuvo en el suelo sin cubrir. Además, las relaciones hídricas de la planta fueron superiores en todos los tratamientos de acolchado, lo que dio lugar a la mejora del rendimiento del cultivo y los parámetros de calidad física más importantes en la alcachofa, a saber, el color, la firmeza y el diámetro ecuatorial. Además, el contenido de algunos nutrientes importantes en la alcachofa (K^+ y N) fue mayor en los tratamientos RH y MS, mientras que las concentraciones de carbohidratos fueron mayores en suelo desnudo con respecto a los otros tratamientos. Los resultados de este primer estudio demuestran que el hidroacolchado mejoró los parámetros fisicoquímicos y biológicos del suelo, induciendo así un mejor estado hídrico de las plantas de alcachofa. Se ha demostrado que esto tiene una implicación directa en la mejora de la productividad del cultivo de alcachofa y las características de calidad física de los frutos de alcachofa. Además, los tratamientos de hidroacolchado, especialmente MS y RH, fueron más sostenibles que el PE en términos de fertilidad del suelo y ciclo de nutrientes, provocando un aumento general en el contenido mineral del fruto de alcachofa, mientras que los carbohidratos disminuyeron con respecto al suelo desnudo. Esto se puede atribuir a una menor retención de agua del suelo desnudo, lo que genera un estrés hídrico transitorio que induce respuestas adaptativas, como la acumulación de azúcares.

Debido a la implicación y a la importancia de los azúcares como parámetros de calidad, además de su papel como osmoprotectores y mensajeros secundarios, se consideró relevante el estudio del metabolismo de la sacarosa bajo condiciones de estrés hídrico en plantas de alcachofa (*Cynara candunculus* var. *scolymus* L.) cv. “Symphony”. Por ello, se realizó un experimento agronómico en condiciones reales de campo para determinar cómo afectaba el estrés hídrico al metabolismo de la sacarosa y la relación fuente-sumidero en plantas de alcachofa. Acolchar el suelo con formulaciones de base orgánica (hidroacolchado) es una alternativa sostenible al acolchado plástico que aquí se plantea como hipótesis para mantener la producción de cultivos bajo estrés por sequía mediante la regulación hormonal y metabólica de las relaciones fuente-sumidero. Para probar esta hipótesis, las plantas de alcachofa (cv. Symphony) se cultivaron en suelo sin acolchado (BS), en suelo acolchado con polietileno lineal de baja densidad (PE), en suelo hidroacolchado con tres mezclas orgánicas diferentes (WS, RH y MS), y se sometieron a regímenes de riego óptimo y reducido un 30% de la evapotranspiración del cultivo. Bajo estrés hídrico, los parámetros de crecimiento fueron más altos en las plantas cultivadas con los diferentes tratamientos de acolchado en comparación con las plantas sin acolchado, lo que se relacionó con una mayor tasa fotosintética y eficiencia en el uso del agua. Es importante destacar que la mejora del crecimiento asociada al acolchado en condiciones de estrés parece estar relacionado con una mayor actividad sacarolítica en las hojas, acompañado de una disminución de los niveles de citoquininas (CKs) activas. Además de esto, el ácido salicílico (SA) disminuyó en las hojas, y el ácido abscísico (ABA) y el precursor de etileno, 1-aminociclopropano-1-ácido carboxílico (ACC) se vieron afectados, lo que se asocia con una mejor regulación de la partición de fotoasimilados.

Dada la relevancia de los resultados obtenidos, en esta tesis se planteó estudiar los efectos del hidroacolchado en un cultivo de ciclo corto de gran relevancia económica, como es la escarola (*Cichorium endivia* L). Se diseñó un nuevo experimento cuyo objetivo fue estudiar los mecanismos fisiológicos involucrados en el control del crecimiento y la productividad mediados por el hidroacolchado de en plantas de escarola sometidas a estrés hídrico, con especial atención al equilibrio hormonal. Se cultivaron plantas de escarola (*Cichorium endivia* L.) cv. "Brillantes" en macetas con sustrato de fibra de coco sin cubrir (BS) o cubiertas con film de polietileno (PE) y tres tipos de hidroacolchados elaborados con aditivos reciclados: paja de trigo (WS), cascarilla de arroz (RH) y sustrato utilizado para el cultivo de champiñón (MS). La mitad de las plantas fueron sometidas una reducción del volumen de agua de riego del 30% de la evapotranspiración del cultivo. A pesar de que el estrés por sequía afectó los parámetros relacionados con el crecimiento de la escarola en todos los tratamientos, las plantas cubiertas con MS mantuvieron un crecimiento significativamente superior, debido a la mejora de las relaciones hídricas de la planta y la función fotosintética. Esto puede explicarse por una interacción eficiente hidroacolchado/sustrato/planta en la regulación del equilibrio hormonal en condiciones de estrés hídrico. De hecho, las concentraciones de las CKs activas transzeatina (tZ) e isopenteniladenina (iP) fueron más altas en las plantas cultivadas con tratamiento MS, lo que se asoció con la mejora del crecimiento de la parte aérea de la planta y el mantenimiento de la tasa fotosintética en condiciones de estrés. Las concentraciones de ABA, hormona relacionada con el estrés, variaron antagónicamente a las de las CKs activas. En este sentido, el ABA aumentó con el estrés hídrico, pero en menor medida en las plantas hidroacolchadas con MS, regulando así la apertura estomática, que, en interacción con el precursor de etileno ACC, mejoraron las relaciones hídricas de la planta.

Como se ha mencionado anteriormente, las técnicas de acolchado se han utilizado tradicionalmente para mejorar el crecimiento de los cultivos. Además, considerando que el hidroacolchado tiene un efecto positivo en la calidad del suelo y en el rendimiento de los cultivos, nos planteamos estudiar su interacción con microorganismos simbiontes como los hongos micorrízicos arbusculares (HMA). Para ello, plantas de escarola (*Cichorium endivia*, L.) cv. "Bekele" fueron inoculadas o no con el HMA *Rhizophagus irregularis* y cultivadas bajo diferentes tratamientos de cobertura: hidroacolchado ecológico basado en sustrato de cultivo de champiñón (MS), polietileno lineal de baja densidad (PE) y plantas no cubiertas (BS). Los resultados han revelado que la inoculación de *R. irregularis* o el uso del hidroacolchado individualmente, pero especialmente su interacción, produjeron una mejora del crecimiento de las plantas. Esta mejora se asoció principalmente a factores hormonales y al aumento de la eficiencia en el uso de NO_3^- y P^{5+}. Las plantas inoculadas cubiertas con MS aumentaron las GAs activas, GA_3 y GA_4, pero especialmente GA_3, que se correlacionaban con todos los parámetros relacionados con el crecimiento estudiados. Asimismo, las concentraciones de CKs, tZ e iP, y ácido jasmónico (JA) aumentaron con la inoculación de HMA o por la aplicación de hidroacolchado, pero particularmente, por su interacción, lo que se asoció con la promoción del crecimiento vegetal. Además, las concentraciones de ACC y ABA disminuyeron en las plantas inoculadas con *R. irregularis* y, especialmente, en las plantas inoculadas y cultivadas con hidroacolchado.

Los resultados de esta tesis son de especial interés ya que el hidroacolchado ha demostrado ser una alternativa sostenible al acolchado plástico para mantener el rendimiento y productividad del cultivo de alcachofa y escarola, con efectos beneficiosos en términos de calidad de suelo, así como su efectividad en la mejora de la respuesta adaptativa de la planta bajo condiciones de estrés hídrico a través de cambios en la homeostasis hormonal de las plantas.

Grounds and objectives

One of the most important problems that limits the sustained development of the agricultural sector in the Mediterranean basin is the scarcity of water resources, as a result of its low annual rainfall. Climate change is aggravating this phenomenon, having an intense impact on productive agricultural activities. Crop yields are expected to decline over the next several decades in most current production areas and for most crops. In addition, the exponential growth of the world population entails a growing demand for food, mostly crop plants that are very demanding in terms of water and nutritional resources, particularly horticultural crops. Artichoke and escarole, which are crop species belonging to the Asteraceae family, are considered two of the most important vegetable crops in the Mediterranean area. Much of the horticultural production is carried out under mulching which has strongly helped to improve crop productivity due to its effect on water retention. The abiotic stresses, such as drought, salinity, and high temperatures, are among the factors that most negatively affect horticultural production in the Mediterranean basin. The exposure to water deficit is expected to increase because of climate change and, together with the low availability of nutrients in the soil, growth and plant development will be severely affected, resulting in a reduction in crop yields and large economic losses. Taking all this into account, a great challenge for breeders and scientists is to develop sustainable agricultural management strategies to maintain or even improve agricultural productivity under conditions of environmental stress, maximizing the efficiency in the use of resources. Therefore, the study of new agrotechnologies that face water scarcity has a great scientific and agronomic value for this Region.

Generally, mulching has been used in agriculture to control the crop environmental conditions at the soil surface and part of the root zone. The advantages of mulching involve earlier and higher yields due to the buffering effect of temperature variations, better control of weed population, and significant savings in irrigation water for horticultural crops. The intensification in the use of plastic materials in agriculture for many decades, even though it has significantly increased crop productivity, has also provoked harmful effects in the agroecosystem. At the end of its useful life, the plastic material used to cover the soil turns into a source of pollution when it is not properly removed or is kept in the soil or burnt. This situation has conducted to the search for ecological alternatives to plastic films. One of the most promising approaches to reduce the use of plastic films is the use of ecological hydromulching, which is a (semi)liquid mulching based on reused crop wastes and other organic materials.

In this thesis, we have studied different hydromulching formulations which have been developed by mixing different organic materials, including paper pulp and crop residues (wheat straw, rice hulls, and a substrate used for mushroom cultivation). These hydromulches solidify on the soil after their application, simulating the effect of traditional mulches on the crop. The use of hydromulches can result in lower water use by preventing soil evaporation, increased soil water-holding capacity, higher nutrient availability due to the decomposition of the hydromulch, reduced water runoff, and protection against certain diseases. The numerous potential benefits have persuaded us to study the effect of hydromulching in horticultural crops, both from an agronomical point of view, considering its effect on soil quality and crop production, and from a physiological and biochemical point of view, studying the mechanisms involved. Furthermore, unraveling the complexities of plant responses to drought stress by using this agrotechnology, could facilitate the development of climate-resilient crops to secure global food production.

Therefore, the general objective of this thesis was to study the effect of the application of new ecological (semi)liquid mulching materials on soil quality and horticultural crop production, as well as to unravel the physiological and biochemical responses to drought stress. To achieve this general objective, the following specific objectives have been set:

I. To evaluate the use of hydromulching in soil physicochemical and biological properties and its influence on yield and quality characteristics of the artichoke crop.

II. To study the hormonal regulation of source and sink relationships associated with the use of hydromulching in artichoke plants subjected to water limitation.

III. To unravel the physiological mechanisms involved in the hydromulching-related growth and productivity control of escarole plants subjected to drought stress, with a special focus on the hormonal balance.

IV. To study the effect of the interaction between hydromulching and arbuscular mycorrhiza fungi in plant growth and productivity of escarole plants.

CHAPTER I

General introduction

1. The climate crisis scenario

Currently, the world has to face the effects of climate change regarding food security by adapting agricultural production to adverse situations. The climate crisis phenomenon is caused by greenhouse gas emissions, such as carbon dioxide (CO_2), that is accumulated in the atmosphere and retain heat, contributing to an increase in the global temperature of the planet. The major effects of climate change are listed as follows: increase in global temperatures, rise in sea level, changes in rainfall patterns, and the expansion of desertification in arid and semi-arid areas are included as the major (Dubrovský et al., 2014). The latest Intergovernmental Panel on Climate Change (IPCC) report indicates that throughout the 21st century, the effects of climate change will reduce economic growth, seriously affecting food security (Pörtner et al., 2022).

Climate is one of the main determinants of agricultural productivity in the world (Kang et al., 2009). Due to the increase in the concentration of greenhouse gases, it is practically unavoidable that there will be changes in climate to which agriculture will have to adapt (Reviewed by Calzadilla et al., 2013; Muhuddin et al., 2013). Importantly, the effects of climate change and their associated impacts will be different depending on the world region. This will require not only changes in the type and mix of crops grown, but also increased investment (McCarl et al., 2016). Beyond the possibilities of adaptation, agriculture is expected to be the sector that will suffer the greatest economic effects from climate change, as Fischer et al., (2005) and Mendelsohn (2009) stated.

1.1 Effects of climate change in the Mediterranean basin

Recently, the IPCC concluded in its Sixth Assessment Report that Europe is highly vulnerable to climate change. Climate models have shown significant agreement for all emission scenarios in warming all over Europe, with the strongest warming projected in southern Europe in summer, and in northern Europe in winter (Forzieri et al., 2016; Kjellström et al., 2011). Climate change also affects precipitation, with regional and seasonal variations. Indeed, precipitations are expected to suffer a sharp decrease in southern Europe, which includes the Mediterranean basin (Kjellström et al., 2011). Indeed, the arid and semiarid regions of the world are particularly affected by water scarcity problems (Dubrovský et al., 2014) due to the limited rainfall, the increase in heat waves, and accelerated soil-moisture evaporation (Cook et al., 2016). This problem has been accentuated by the rapid rise of the world population, global warming, and climate change situation, which increases pressure on limited water resources (Çolak et al., 2022). According to the climate models projections stated by the Mediterranean Experts on Climate and Environmental Change (MedECC, 2020), the climate in the

Mediterranean basin is changing, historically, faster than global trends. As Figure 1 shows, mean annual land and sea temperatures in the Mediterranean basin are 1.5°C higher than in pre-industrial times and are projected to rise an additional 3.8-6.5°C by 2100 under a high concentration scenario of greenhouse gases (RCP 8.5), and between 0.5 and 2.0°C in a scenario compatible with the long-term objective of the UNFCCC Paris Agreement of keeping the global temperature well below +2°C above pre-industrial level (RCP 2.6).

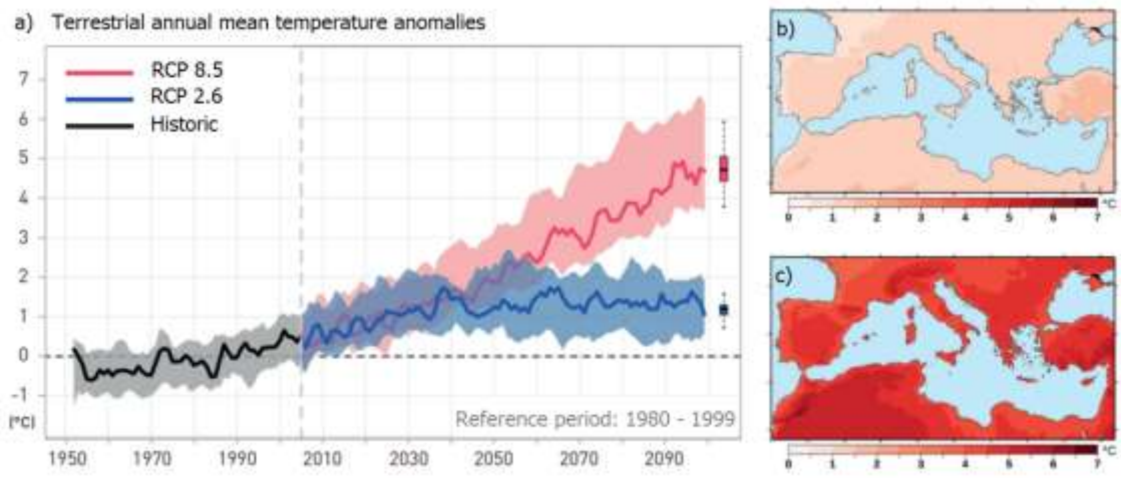

Figure 1. Expected warming in the Mediterranean basin. The changes expected in annual temperature related to reference recent past period (1980-1999), based on the mean of EURO-CORDEX dataset; a) simulations for the RPC 2.6 and RPC 8.5; b) warming in the end of XXI century (2018-2090) for RCP 2.6; and c) warming at the end of XXI century (2018-2090) for RCP 8.5. Adapted from MedECC (2020).

Agriculture is the main user of water in the world. Climate change affects water resources in combination with demographic and socioeconomic factors, reducing runoff and groundwater recharge, water quality, ecosystem degradation, and salinization of water resources (Cramer et al., 2018). Regarding the effects of climate change on rainfall, according to MedECC (2020), it is expected a constant decrease in rainfall during the 21st century for the entire Mediterranean basin during the warm season and in winter for most of the Mediterranean area (Figure 2). Global warming, the decrease in mean rainfall, and the increase of atmospheric evaporative demand produce an ideal scenario for plant water stress, leading to agricultural and ecological drought (Pörtner, 2022). Drought is considered the main threat to food production through crop damage and yield decreases (Tigkas et al., 2019). In this context, it is highly prospective that crop yields will decrease significantly. Consequently, farming practices in these areas are being developed in order to conserve soil moisture.

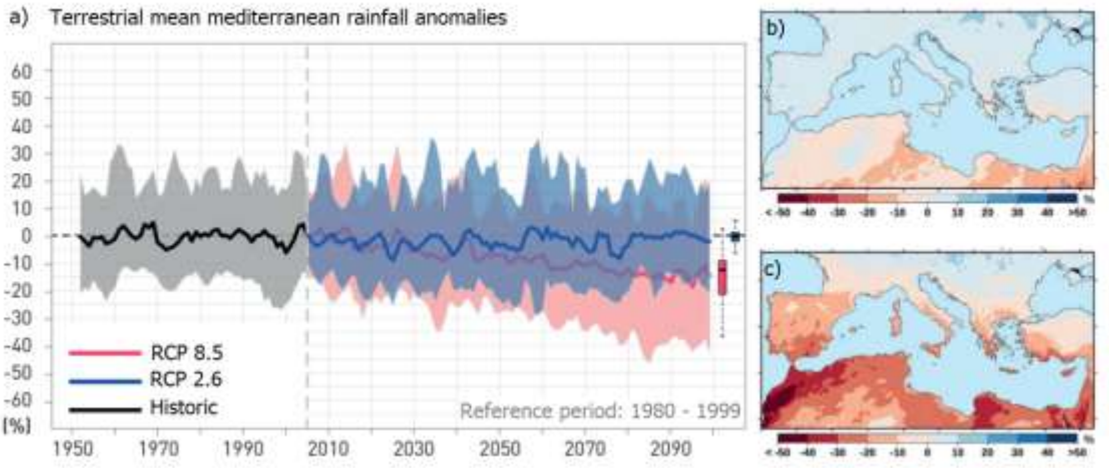

Figure 2. Expected rainfall changes in the Mediterranean basin. The changes expected in annual rainfall related to reference recent past period (1980-1999), based on the mean of EURO-CORDEX dataset; a) simulations for the RPC 2.6 and RPC 8.5; b) warming at the end of XXI century (2018-2090) for RCP 2.6; and c) warming at the end of XXI century (2018-2090) for RCP 8.5. Adapted from MedECC (2020).

1.2 The effects of drought on the plants

Environmental stress is defined as an external factor that produces a negative influence on the plants, producing unfavorable situations for their development and optimal functioning. In plant physiology, these unfavorable conditions are known as "stress" and it produces several important physiological, biochemical, and metabolic changes and adjustments. Stress can be produced by pathogenic organisms, which is defined as biotic stress, and it can be accentuated due to the climatic conditions in which the infection occurs.

Additionally, the most common abiotic factors that affect crop production are essentially physical factors, such as deficit or excess of water, extreme temperatures, salinity, and chemical factors, such as the deficit or excess of certain nutrients. Due to the current climate change crisis, these stressors are rapidly spreading or aggravating, which entails a major threat to the world's future food security. Drought and, to a lower extent, extreme temperatures are the most common abiotic factors that affect seriously plant growth and development and therefore crop production (Lamaoui et al., 2018). In the Mediterranean basin, the most important stress factor is water deficit, which is interrelated with other stressors such as high temperatures or salinity (el Sabagh et al., 2019). In fact, yield decrease caused by these factors, particularly water stress, has not ceased to increase in recent decades, causing concerns in the agricultural systems. As has been stated above, the latest IPCC report aims to a worsening of the effects of stress in the coming decades, with their consequent impact on crop yields.

Water is the essential molecule for life and is considered the most abundant and best-known solvent. Particularly in plants, water typically constitutes 80% to 95% of the mass of growing tissues and performs several unique functions. Due to its polar properties, it has a great influence on the structure and stability of molecules such as proteins, polysaccharides, and others (Kirkham, 2014). Likewise, cell expansion and the physical-chemical integrity of the cell wall depend on water. A list of processes that are regulated by cell volume and hydrodynamics includes, in addition to those mentioned, growth and proliferation, exocytosis, endocytosis, changes in cell shape, hormone signaling, metabolism, excitability, cell migration, nutrients obtaining, debris filtration, necrosis, and apoptosis (Guo et al., 2017; Kieran et al., 2000). Considering the great importance of water in plants, it can be taken into account that a limited or excessive amount of water for them constitutes an inducing factor of adverse or stressful situations.

In a situation of water scarcity, plants suffer a massive reprogramming of the metabolism in which cell survival becomes the main priority taking away resources destined for growth (Reviewed by Mickelbart et al., 2015), thereby making drought one of the main limiting factors of plant productivity (Claeys & Inzé, 2013). From an agricultural point of view, the stress provoked by drought is caused by low soil moisture that limits water availability in the root zone, negatively affecting plant growth and development, and therefore decreasing crop productivity (Yadav et al., 2020). Plants use a multitude of adaptive mechanisms to combat the effects of water stress (Dodd & Ryan, 2016). Simplifying, we can classify these mechanisms into three main groups:

- *Dehydration escape mechanisms:* Plants adapt their phenology to the availability of water in the environment so that they are not affected by times of drought. Although these mechanisms are of great ecological importance (Kooyers, 2015), they also are agronomically relevant, especially in annual cycle crops.

- *Dehydration avoidance mechanisms*: Plants use different mechanisms to maintain their hydric status during times of drought, attenuating to a greater or a lesser extent its effects. One of the most common avoidance mechanisms is the restriction of water loss through transpiration by reducing leaf production and slowing their growth (moderate drought) or accelerating plant senescence (severe drought). Prolonged episodes of drought can reduce the total number of leaves or the leaf area, with a consequent impact on the photosynthetic activity. The stomata closure is one of the main plant strategies to avoid water loss by transpiration and it is mainly regulated by the hormone ABA (Sah et al., 2016). The goal of stomatal closure due to water deficit is to increase transpiration efficiency (Figure 3). However, stomatal closure also leads to a decrease in CO_2 absorption, which can be counterproductive if we refer to the increase in photosynthetic efficiency that stomatal closure pursues. These mechanisms logically decrease the global photosynthetic activity of the plant and therefore negatively affect crop productivity (Chaves et al., 2002).

- *Dehydration tolerance mechanism*: These mechanisms are associated with rapid responses after the perception of stress in order to maintain cellular homeostasis and concentrate practically all of the genetic modifications tested so far (Todaka et al., 2015). Among the most studied mechanisms of this type, the reviews of Claeys & Inzé, (2013) and Tenhaken (2015) stand out the synthesis of osmoprotectants, also called compatible solutes, such as proline or certain monosaccharides, whose role is to modify significantly the intracellular water potential, as well as the synthesis of protective proteins, such as aquaporins (water transport), molecular chaperones (stabilizing proteins and membranes), or detoxifying enzymes ROS (antioxidant capacity). On the other hand, the mechanisms to tolerate desiccation or loss of water in the cell include a change in hormonal biosynthesis which are hugely relevant (Figure 3).

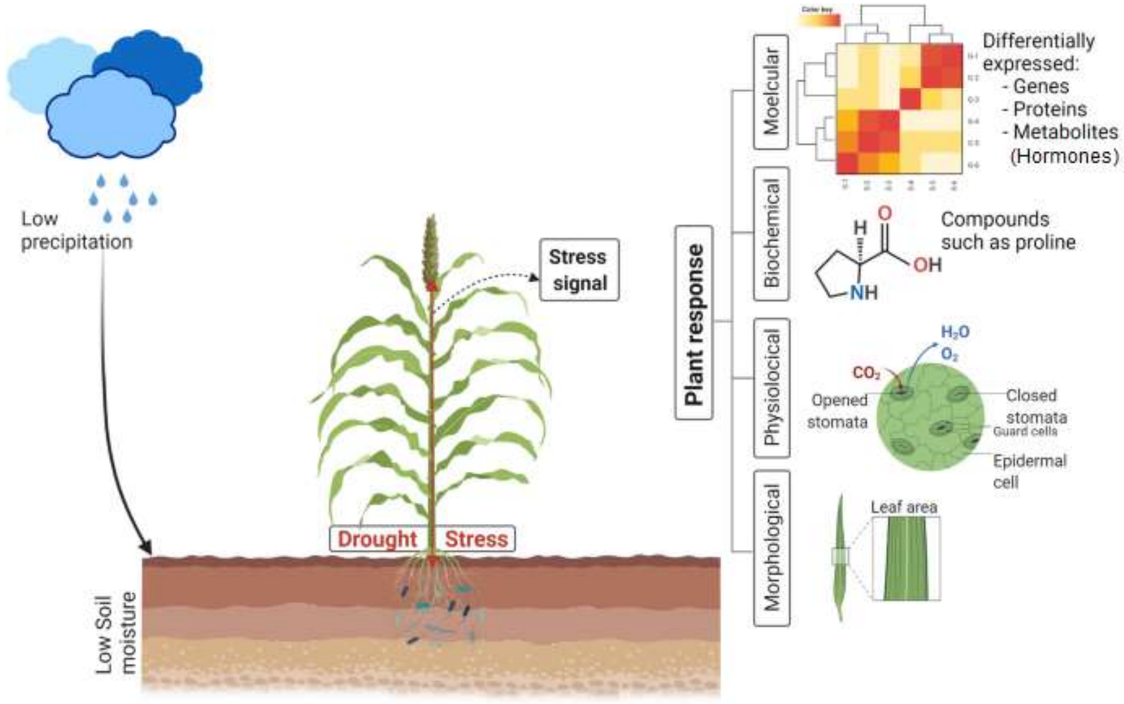

Figure 3. Diagrammatic depiction of morphological, physiological, biochemical, and molecular responses of plants to drought stress. This figure was adapted from Abreha et al. (2022).

2. Phytohormones and their implication in water stress responses

The mechanisms described above, which allow plants to survive water stress situations, are orchestrated mainly by phytohormones which are considered crucial endogenous substances for modulating physiological and molecular responses for plant survival (Ullah et al., 2018). Indeed, phytohormones play a key role in the control and regulation of the physiological mechanisms of the plant, both under favourable and constraining conditions (Zhao et al., 2021). Phytohormones are a type of chemical signal, whose function is to coordinate the activity as well as facilitate intercellular communication and the proper growth of the plant. They have widely overlapping functions and therefore the hormonal regulation that occurs during plant development is produced as crosstalk between different phytohormones (Diopan et al., 2009; Liu et al., 2017). Plant cells, respond through a stimulus-response coupling mechanism to hormonal signals, which requires a receptor to recognize the hormone, as well as the involvement of signal-transmitting molecules that can induce the activation of a specific response, followed by the signal transduction cascade of the hormonal signal (Gaspar et al., 2003).

2.1 Phytohormone classes

Each phytohormone has specific functions that depend on the endogenous levels of the active hormone. These levels are mediated by the balance between biosynthesis, catabolism, and conjugation of the hormone itself (Diopan et al., 2009). In addition, plant hormones interact with each other antagonistically, synergistically, or additively, forming a complex network of hormonal signaling (Gazzarrini & McCourt, 2003; Jaillais & Chory, 2010; Peleg & Blumwald, 2011).

2.1.1 Abscisic acid

Abscisic acid (ABA) is widely known as the “stress hormone” since is considered the central regulator of the resistance mechanisms against abiotic stress in plants (Kuromori et al., 2018). ABA is a sesquiterpenoid derived from zeaxanthin, predominantly synthesized in apical root cells but also in mesophyll cells of the leaves (Achard et al., 2006; Silva et al., 2018). Harsh environmental conditions induce ABA biosynthesis, whose endogenous concentration decreases when stress is relieved (Ullah et al., 2018).

Although initially its name indicated that it was involved in the abscission of fruits, leaves, and flowers (Schwartz & Zeevaart, 2010), ABA is a hormone that regulates the dormancy of seeds and buds, in addition to actively participating in multiple physiological processes for plants, such as embryo maturation and germination, inhibition of division and cell elongation, promotion of stomatal closure (Eyidogan et al., 2012; Montillet et al., 2013), floral transition (Wang et al., 2013), synthesis of storage proteins and lipids, and especially, in processes of response to various types of abiotic stress as drought, salinity, cold, and ultraviolet radiation, among others (Finkelstein et al., 2002; Kuromori et al., 2018; Li et al., 2021). Despite being classically known as a growth-inhibiting hormone, ABA maintains an important role in the maturation of non-climacteric fruits, since it acts as a regulator of the change of color and flavor of the fruits, through the synthesis of anthocyanins and the accumulation of sugars, which occurs at the beginning of the maturation process (Bai et al., 2021; Li et al., 2011; Ren et al., 2011). Furthermore, several authors have also informed the role of ABA in plant defense mechanisms against pathogens (Sánchez-Vallet et al., 2012).

The role of ABA in the mechanisms of tolerance to abiotic stress of the plant has been extensively studied (Li et al., 2021; Sah et al., 2016; Saradadevi et al., 2017). In contrast to other hormones, ABA acts as a stress signal to adjust plant growth in response to changes in water availability. The main function of ABA seems to be the regulation of plant water balance and osmotic stress tolerance (Tuteja, 2007). During drought stress conditions, ABA is accumulated in plant cells producing an alteration of gene expression (O'brien et al., 2013), thus acting as a controller in plant tolerance and the response to stress (Reviewed by Vishwakarma et al., 2017). Under water deficit, ABA levels are increased in both leaves and roots to reduce transpiration and induce the synthesis of osmoprotective molecules and proteins to increase resistance to desiccation (Tseng et al., 2013). Indeed, in a situation of limited water availability, ABA is first synthetized in the root and sends a signal to the shoot so that water loss through transpiration is minimized by the decreased expansion of leaves and most importantly due to the process of stomata closure (Wilkinson et al., 2012).

Stomata closure is considered one of the most important responses to water stress to prevent water loss by transpiration. Several studies conducted in various agronomic species have confirmed an increased concentration of ABA in leaf tissues associated with reduced stomatal conductance under water deficit (Li et al., 2021; Saradadevi et al., 2017; Xu et al., 2018). Stomatal movements are mediated through cell turgor, which is controlled by the transport of ions across membranes (Ullah et al., 2018). During water stress exposure, the ABA content in the leaf increases due to the decompartmentalization and redistribution from the chloroplasts of the mesophyll cells and to the synthesis and transport from the roots, being released to the apoplast to reach the guard cells through the transpiration current (Hartung et al., 2002), affecting seriously the photosynthetic apparatus. Din et al. (2011) observed a sharp decline in the levels of chlorophylls in plants upon exposure to drought stress, which was attributed to the negative effect on enzymes related to chlorophyll biosynthesis. ABA produces a loss of K^+ and Cl^- in the guard cells, which causes an outflow of water from the cytoplasm, leading to a drop in water potential (Misra et al., 2015). When the water potential in leaf cells decreases, ABA causes stomata closure, which reduces water loss via transpiration. Stomatal regulation to limit water use during water deficit is at the expense of CO_2 diffusion into leaf tissue, which subsequently leads to decreased CO_2 intake, affecting the rate of photosynthesis (Chaves et al., 2002) and altering photosynthetic metabolism (Lawlor & Cornic, 2002). ABA also induces the expression of many genes whose products are very important for stress responses and stress tolerance, such as enzymes for osmoprotectant synthesis and antioxidant enzymes, which provides tolerance against desiccation (Reviewed by Fujita et al., 2011).

2.1.2 Cytokinins

Cytokinins (CKs) are a group of phytohormones that are transported ascending throughout the plant and responsible for promoting or stimulating cell division and development (Mok, 2019). In 1963, the first natural CK was isolated from maize plants (*Zea mays* L.) and was named zeatin, which is considered the most abundant CK in plants (Letham, 1973). Natural CKs can be structurally defined as adenine-derived molecules with a side chain attached to amino group 6 of the purine ring. There exist two main types of CKs, zeatin type (Z-CKs) and isopentenyladenine type (iP-CKs). The side chain may be isoprenoid or aromatic in nature. Among the isoprenoid CKs are isopentenyladenine, zeatin, both *cis* and *trans*, and dihydrozeatin, with their respective ribotides, ribosides and riboside glycoside derivatives. Free forms, such as trans-zeatin (tZ) and isopentenyladenine (iP), are considered the active forms (Sakakibara, 2006).

Cytokinins are present in the xylem and phloem, and along with auxins, they act in the G1 phase, inducing storage and accumulation of cyclins, and promoting a new cell cycle (Smith & Atkins, 2002). They are also responsible for the partial or total nullification of the apical dominance promoting growth and budding of axillary buds, and can form adventitious shoots on veins, petioles, and leaf parts (Howell et al., 2003). The CKs take action, among other functions, in the regulation of root structure (Bianco et al., 2013) and slow down the process of senescence (Zwack & Rashotte, 2013). They also participate in anthocyanin production and photomorphogenesis, leaf expansion through cell enlargement, stomatal opening, and chlorophyll production (Cortleven & Schmülling, 2015; Davies, 2004; Wu et al., 2021).

Endogenous levels of CKs are good indicators of stress responses, which greatly change with the severity of the stress (Reviewed by Bielach et al., 2017). They are involved in establishing tolerance under drought conditions (Hai et al., 2020) and under high salinity and high temperatures (Javid et al., 2011). In drought situations, CK synthesis in the roots is altered, resulting in a decrease in their transport to the shoot and altering the expression of certain genes, which could cause a series of physiological responses to combat stress (Nishiyama et al., 2011). This indicates that the levels of CKs act as a limiting factor aimed at favouring tolerance to osmotic stress. In addition, (Akter et al., 2014) informed that the exogenous application of CKs and gibberellic acid in wheat-stressed plants were found to be very effective in alleviating drought-imposed adverse effects on plants at the vegetative phase. Cytokinins are considered antagonists of ABA in different processes, such as the stimulation of stomatal opening and the increase in the rate of transpiration. After numerous studies, it has been confirmed that ABA and CKs interact in such a way that the action of ABA to maintain the closed stomata would complement CKs, favouring delayed stomatal closure and leaf senescence (Pospíšilová et al., 2005).

2.1.3 Gibberellins

The origins of gibberellins (GAs) date back to the 19th century. The disease called bakanae in rice plants (*Oriza sativa* L.), caused by the fungus *Gibberella fujikuroi*, produced a substance that promoted an excessive elongation of seedlings (Yabuta et al., 1934). These growth-causing plant substances were called GAs after the first studies on their properties. Owing to the studies with dwarf mutants of *Pisum sativum* L. and *Zea mays* L., and their properties as growth promoters, GAs were recognized as phytohormones (Hedden & Sponsel, 2015). GAs are part of a relatively large family of acid diterpenes whose function consists in the regulation of plant growth and development (Davies, 2004). GAs are presented at concentrations in high buds and leaves and fruits that are actively growing. The synthesis of GAs can occur in many places in the plant and is regulated by environmental and endogenous stimuli (Davière & Achard, 2013).

This group of hormones participates in inducing flowering, by activating signals that target meristematic genes intended to be differentiated into stamens, petals, carpels, etc. (Yu et al., 2004). They also play a fundamental role in the mobilization of reserves, which occurs in cereal grains, especially in the induction of the synthesis of proteases and α-amylases, also favouring parthenocarpy and the development of multiple fruits (García-Hurtado et al., 2012; Nanjo et al., 2004). Due to its involvement in various biological processes, GAs are commonly used in compounds for commercial and biotechnological fields such as horticulture and floriculture (Salazar-Cerezo et al., 2018).

Several studies have shown that, in plants subjected to abiotic and biotic stresses, the levels of biologically active GAs are clearly diminished (Colebrook et al., 2014; Javid et al., 2011). Importantly, phytohormones crosstalk with others in adverse situations to favour the control of plant growth and development. The study of Shu et al. (2015) informed that under osmotic stress conditions those known as proteins DELLA, involved in GAs signaling, act together with other hormones to adapt to unfavourable environments. Gibberellins along with CKs are commonly considered to act as ABA antagonists (Acharya & Assmann, 2009; Shu et al., 2015). It is known that the synthesis of GAs increased in ABA-deficient-2 mutants of Arabidopsis, indicating that ABA is involved in the suppression of GA synthesis and that the transcription factor ABI4 may be the molecular factor that modifies the balance of ABA and GA biosynthesis (Liu & Xingliang, 2018).

2.1.4 Auxins

At the beginning of the 20th century, it was identified a growth-promoting compound in the coleoptiles of *Avena sativa* L. (Went, 1942) named auxin, which becomes from the Greek word auxein, whose meaning is "grow". Its chemical structure was subsequently identified as indole-3-acetic acid (IAA). Auxins were the first phytohormones to be discovered and have a major role in plant morphogenesis, including shoot growth, patterning roots, vascular tissue differentiation, and formation of auxiliary and floral buds (Sauer et al., 2013). Indoleacetic acid is considered the main auxin in most plants, which is a derivative of the amino acid tryptophan (Davies, 2004).

Along with GAs and CKs, auxins regulate multiple physiological processes in plants related to growth and development. Auxins are located in areas of active growth as young leaves, apical meristems, fruits and seeds in development, as well as in xylem, cambium, and phloem. One of their main functions is cell elongation and regulating the permeability of the cell membrane. They are also responsible for apical dominance and the cell division of the cambium cells (Oles et al., 2017; Rashotte et al., 2003). It has been observed that IAA delays floral senescence and fruit ripening, regulates abscission, and promotes femininity of dioecious flowers, by interacting with ethylene. Indoleacetic acid also regulates the expression of a large number of defense genes, and mediates interactions between abiotic and biotic stress responses (Davies, 2004).

The endogenous levels of auxins are affected by water stress causing a reduction in stem growth and development, since the biochemical cascade triggered by ABA affects auxin concentrations (Popko et al., 2010). It is the case, for example, of the ARF2 gene induced by ABA, which influences negatively in the transcription of certain genes that are expressed in response to auxins (Lim et al., 2010). Abscisic acid inhibits the action of auxins, reduces or even avoids the loss of water in the foliar zone by stomatal closure, and has a role in the reorganization of root growth (Popko et al., 2010). In these osmotic stress conditions, there exists an increase in

ABA levels, which causes auxins to be transported to the root apex, being stored and redistributed in them (Xu et al., 2013). Auxins, once in the root, induce the secretion of H^+, promoting an increase in H^+ activity-ATPase located at the level of the plasma membrane, which is essential for the development of root hairs and produces the phenomenon of primary root elongation (Santi & Schmidt, 2009). Several lines of experimental evidence have also shown that auxins, which are hormones normally associated with root initiation and growth, increase tolerance to water stress. An increase in the levels of IAA and/or in indole-3-butyric acid (IBA) in overexpressors Arabidopsis mutant plants increased growth and plant survival under water deficit, thus the manipulation of auxin homeostasis might be a new avenue in crop protection for water stress tolerance (see for example Tognetti et al., 2010).

2.1.5 Ethylene

Ethylene was the first gaseous hormone to be discovered in plants. Yang and Hoffmann published the methionine cycle or "Yang cycle", from which ethylene is synthesized from methionine through the intermediate 1-amino-1-cyclopropane carboxylic acid (ACC), its precursor (Yang, 1986). Ethylene can act from seed germination to the phenomenon of senescence, which involves all stages of the development of the plant, participating in conjunction with other hormones (Iqbal et al., 2017). This hormone is involved in processes such as seed germination, development and differentiation of stems and roots, flowering, abscission of leaves, flower senescence, and fruit ripening (Lin et al., 2009). Indeed, due to its key role in plant senescence and fruit ripening, ethylene is widely known as the “fruit ripening hormone” or the “aging hormone” (Barry & Giovannoni, 2007). Therefore, it is usually used as an agent promoter of fruit ripening for commercial purposes.

In stressful situations, ethylene plays a fundamental role in the adaptation and survival to adverse conditions either by activating mechanisms of signaling or by being the end effector of the response (Bleecker & Kende, 2000). It has been demonstrated that under drought conditions, ethylene caused leaf abscission and consequently reduced water loss (Arraes et al., 2015). Stomatal closure is a key response for plant abiotic stress tolerance, and ethylene has been shown to act antagonistically to ABA, by inhibiting stomatal closure through a complex signaling network (Reviewed by Müller & Hasanuzzaman, 2021). There might be some correlation between ethylene synthesis and the regulation of osmolyte accumulation that can protect the plants under several stressful conditions (Reviewed by Sharma et al., 2019). The study of Iqbal et al. (2012) and (2014) informed about the implication of ethylene in the regulation of abiotic stress through its impact on osmoprotectant accumulation. CKs establish a crosstalk with ethylene due to their relevant implication in the regulation of the Mitogen-Activated Protein Kinase 6 (MAPK6) pathway, an important part of ethylene cell signaling (Ouaked et al., 2003), which is considered to play an important role in biosynthesis and accumulation of osmolytes in plants (Reviewed by Khan et al., 2017).

2.1.6 Jasmonic acid

In 1962, the methyl ester of jasmonic acid (JA) was first isolated (methyl jasmonate, MeJA) from an essential acid from the *Jasminum grandiflorum* L., or jasmine (Demole et al., 1962). It was in the early 1990s when JA was demonstrated as a powerful signal in plant defense against pathogens, and its involvement in numerous physiological processes (Browse, 2009). According to the review of Ahmad et al. (2016), JA is the main organic compound of the group of phytohormones called jasmonates, which is involved in signal transmission produced by wounds and pathogens, affecting numerous physiological processes. The main function of JA and its various metabolites is to regulate plant responses to abiotic and biotic stresses, as well as plant growth and development (Delker et al., 2006). Among its most notable effects, it can be highlighted that it is a promoter of the formation of adventitious roots, chlorophyll degradation, and it is involved in the ripening and color of the fruits since it induces ethylene synthesis (Soto et al., 2012).

It is widely known that JA induces plant disease responses against pathogens (Ahmad et al., 2016). These responses include the production of secondary metabolites and protein expression involved in defense response, including alkaloids, terpenoids, phenylpropane, amino acid derivatives, anti-nutritional proteins, and some pathogen-related proteins (Fragoso et al., 2014). Although traditionally the JA-mediated responses to biotic stress have been extensively studied, its implication in abiotic stress responses is currently being studied in greater detail (Attaran et al., 2020; Zhen Luo et al., 2019). The application of exogenous JA and salicylic acid (SA) alleviated the damage caused by osmotic stress in maize crop by increasing protective osmolytes, including proline, total carbohydrate content, and total soluble sugars (Tayyab et al., 2020). On the other hand, JA and the JA precursor 12-Oxophytodienoic acid (12-OPDA) are related to the promotion of stomatal closure. The study of Savchenko et al. (2014) informed that the increase of 12-OPDA content was related to the decrease of stomatal conductance, thus improving drought resistance in Arabidopsis plants. Moreover, some antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) were increased and subsequently enhanced the ability of plants to cope with drought stress (Sarwat & Tuteja, 2017; Tayyab et al., 2020). Furthermore, the transitory accumulation of JA during water stress appears to be required for ABA biosynthesis in response to drought (de Ollas et al., 2013).

2.1.7 Salicylic acid

Salicylic acid is part of a large group of compounds synthesized in plants called phenolics, which have in their chemical structure a hydroxyl group attached to an aromatic ring. Salicylic acid comes from the amino acid phenylalanine, and it is an important phenolic compound at the level of the regulation of plant growth and plant immunity (An & Mou, 2011; Dempsey et al., 2011). This hormone participates in processes such as seed germination, cell growth, stomatal closure, expression of genes associated with senescence, response to abiotic stress, and it is essential in thermogenesis as well as in the resistance to diseases (Reviewed by Vlot et al., 2009). Additionally, it has been described that, in some cases, the effect of SA within the plant metabolism can be indirect since alters the synthesis and/or signaling of other hormones including JA, ethylene, and auxins (Ahmed et al., 2020; Balbi & Devoto, 2008).

Salicylic acid is widely known for its response to biotic stress. However, several studies have also confirmed its implication in the response of plants against abiotic stresses such as drought. The study of Habibi (2012) stated that the foliar SA supplementation resulted in increased net CO_2 assimilation rate due to higher stomatal conductance during an episode of drought in a barley crop. Other studies informed that exogenously applied SA can modulate important enzymatic and non-enzymatic antioxidants and decrease oxidative stress in drought-exposed plants (Reviewed by Sharma et al., 2019). Furthermore, SA is also implicated in the modulation of osmolyte synthesis, such as proline and sugars, under drought (Tayyab et al., 2020; Yuan et al., 2014). Figure 4 summarizes the main mechanisms triggered by plants to respond to water stress conditions which are regulated by hormones.

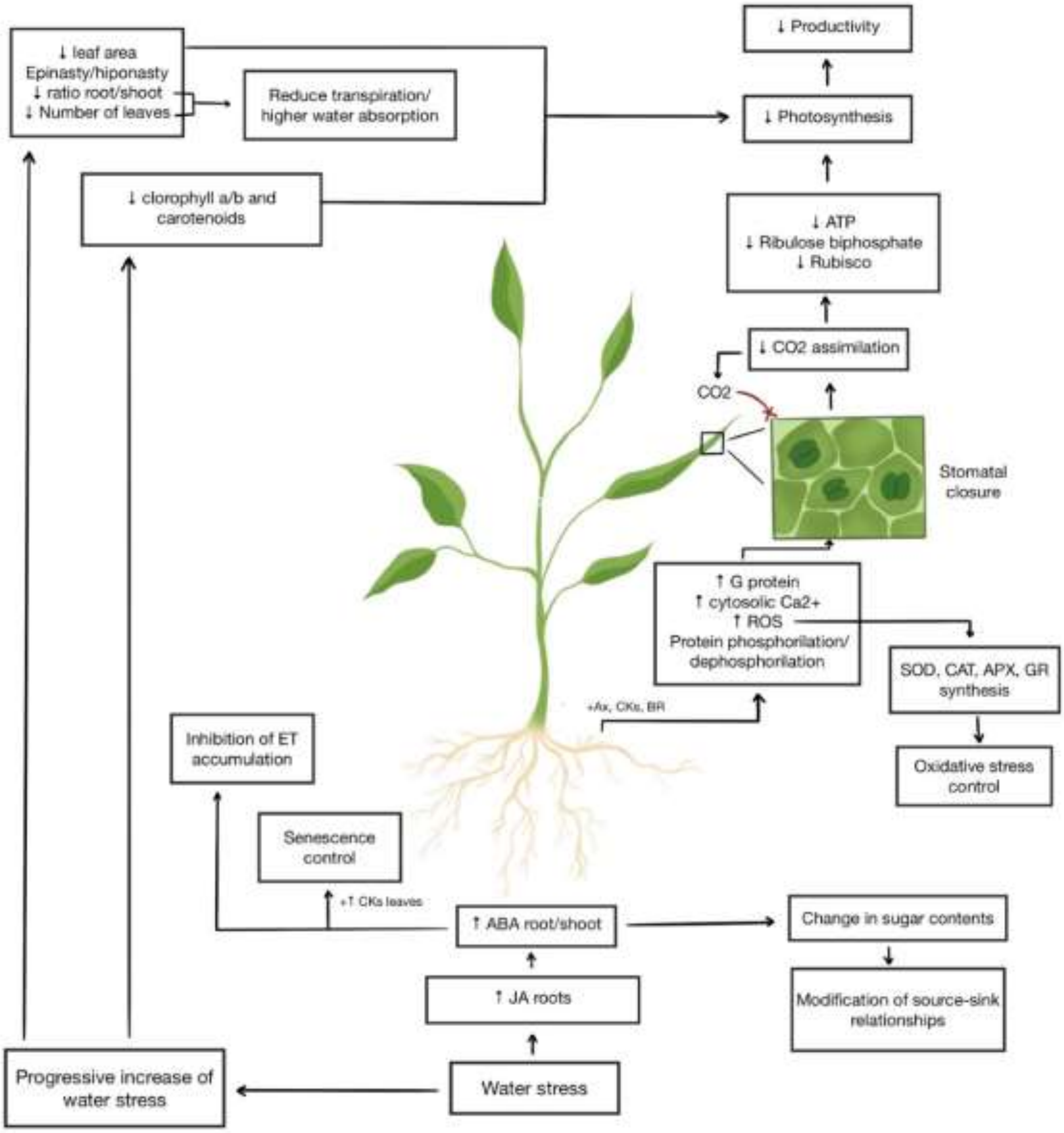

Figure 4. Scheme of plant responses to water stress. Adapted from de Oliveira et al. (2013). Abbreviations used: ABA (abscisic acid), APX (ascorbate peroxidase), Ax (auxins), BR (brassinoesteroids), CAT (catalase), CKs (cytokinins), ET (ethylene), JA (jasmonic acid), ROS (reactive oxygen species) and SOD (superoxide dismutase).

2.2 Phytohormone-mediated regulation of sucrose metabolism: a crucial process in plant productivity

One of the most common physiological responses of plants to water stress, regardless of the species, is the closure of the stomata (Flexas & Medrano, 2002). As has been aforementioned, the process of stomatal closure when the mesophyll begins to dehydrate is regulated by ABA. Stomatal closure produces a reduction in photosynthesis and, therefore, affects carbon metabolism (Chaves et al., 2009; Muller et al., 2011). A carbon deficit could be expected to occur under these conditions. However, the literature shows that carbon compounds often accumulate in different plant organs. Such accumulation has been observed in several crop species, different plant organs, and for soluble or structural carbon. Soluble carbohydrate accumulations have been described both in the leaves of several crops (Gulati et al., 2014; Liu et al., 2004; Su et al., 2022) and in other plant organs as roots and fruits (Vaillant-Gaveau et al., 2014; Xu et al., 2015). This sugar accumulation occurs both after rapid osmotic shocks and during prolonged exposure to water deficit (Gupta & Kaur, 2005), since they are considered compatible solutes because they can accumulate in large amounts without disturbing cellular functions. Soluble sugars are thought to protect subcellular structures against the deleterious effects of water loss from the cell.

In most plants, the disaccharide sucrose is the main end-product of photosynthesis. Sucrose is synthesized in mature leaves (source organs) and translocated to sink tissues as young leaves, fruits, or roots via the phloem to support heterotrophic metabolism and growth, or to be stored as sucrose or starch. Plant growth and development are accompanied by changes in source-sink relationships (Roitsch & González, 2004). Sucrose metabolism is considered one of the key regulatory systems in drought tolerance (Bonfig et al., 2010), playing important roles in plant development, yield, and stress responses (Balibrea et al., 2000; Ruan, 2014). The sucrose hydrolysis in sink organs demands cleavage of the glycosidic bond, catalyzed by sucrolytic enzymes, mainly the invertases (Reviewed by Ruan, 2014). Invertases are a group of key enzymes that can hydrolyze sucrose into glucose and fructose (Tymowska-Lalanne & Kreis, 1998) and play vital roles in providing carbon to plants and major roles in sugar signaling and tissue development (Chen et al., 2022). According to Roitsch & González (2004), in higher plants there exist three types of invertases differentiated by their subcellular localization, pH optima, and isoelectric point: vacuolar invertase (vacInv), cytoplasmic invertase (cytInv), and cell-wall bound invertase (cwInv). Vacuolar invertase and cwInv are glycoproteins that share several biochemical properties, for example, they have an optimum pH between 4.5 and 5.0 and attack the disaccharide through fructose. Therefore, they are also called acid invertases and are β-fructofuranosidases, which also hydrolyze other fructose-containing oligosaccharides, such as raffinose and stachyose. The cytInv is not glycosylated and due to its optimal neutral or slightly alkaline pH, it is also known as neutral or alkaline invertase. In contrast to acid invertases, cytInv specifically attacks sucrose (Huang et al., 2007; Roitsch & González, 2004). Invertases play a key role in various aspects of the plant life cycle and in response to environmental stimuli, since their substrates and reaction products are nutrients and signaling molecules (Roitsch & González, 2004; Sturm, 1999). Abiotic stresses modify source-sink relationships that influence plant growth as well as adaptation to stress and, consequently, affect crop productivity. Although little is known about the mechanisms responsible for growth reduction under water or salt stress conditions, tolerance to abiotic stresses and crop productivity depend on the ability of the plant not only to supply resources to the growing sink tissues, but also to maintain the production of assimilates in mature leaves through delayed senescence (Albacete et al., 2015; Balibrea et al., 2000, 2003; Balibrea et al., 2004). Several studies have reported that under water stress differences in storage carbohydrate accumulation were correlated with differences in sugar profiles, expression of invertase genes, and levels of fructan biosynthesis (Reviewed by Albacete et al., 2014). Saeedipour & Moradi (2011) found that invertases in flag leaves and grains induced by water deficit were responsible for yield stability in a tolerant cultivar of wheat.

Other authors have shown a direct correlation between plant hormones in sink-related processes and their effects on invertase gene expression (Yu et al., 2015). Furthermore, a recent study has shown a positive correlation between the expression of the vacInv gen *CsVI2* and vacInv activity induced by drought in cucumber seedlings (Chen et al., 2022). In another study, Trouverie et al (2003) observed a strong enhancement of vacInv activity that appeared to be highly correlated with xylem sap ABA concentration due to the stomatal closure in maize mature leaves subjected to water deficit. Albacete et al (2015) discovered a plant mechanism of adaptation to drought stress markedly improved in tomato plants due to the overexpression of the cwInv gene *CIN1* together with increases in the senescence-delaying hormones CKs and decreases in the senescence-inducing ethylene precursor ACC. Indeed, plant hormones are widely known to influence growth differentiation and they have been proposed as specific plant growth regulators particularly involved in regulating sink strength and photosynthate partitioning (Roitsch & Ehneß, 2000; Yu et al., 2015). Classically, ABA has been considered as a stress-related hormone since it enhances drought tolerance in plants by means of various physiological and molecular factors, including stomata regulation, root development, and initiation of ABA-dependent pathways (Albacete et al., 2009). Studies performed on rice and wheat have stated that the overexpression of ABA biosynthesis and signaling regulators provoked overall improvements in source and sink capacity due to the stomatal closure, maintaining photosynthetic efficiency and converging into an improvement of yield and drought tolerance. Figure 5 shows a scheme of the hormonal and metabolic regulation of source-sink relationships in plants.

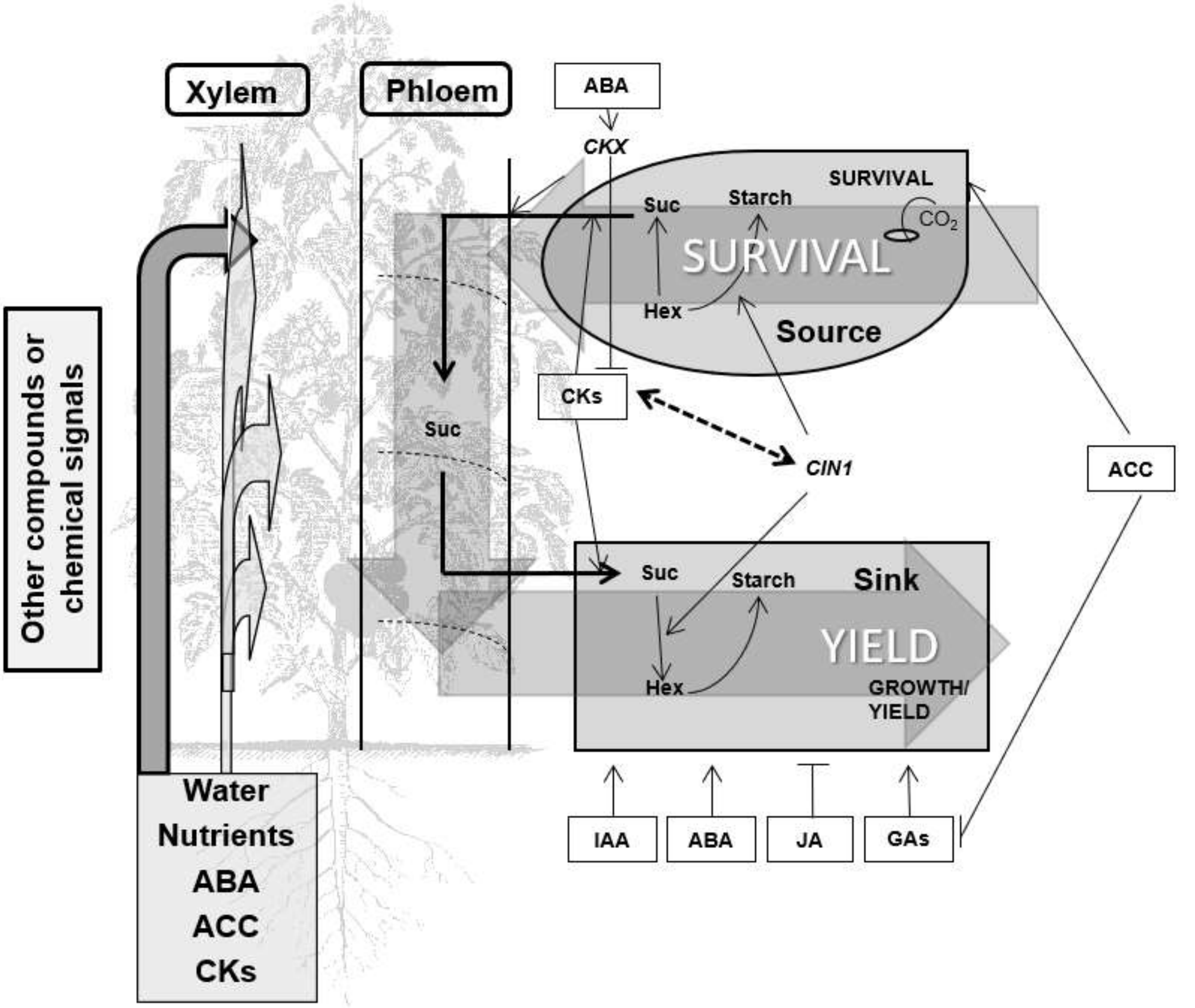

Figure 5. Interactions between hormones and carbon metabolism in the regulation of source and sink activities, phloem assimilate (un)loading and transport, and their relative influence on plant survival (only source activity is required) and on growth/yield (sink activity and transport from the source are also required) related processes under drought conditions. From Albacete et al. (2014). Abbreviations used: ABA (abscisic acid); ACC (1-amino-cyclopropanecarboxylic acid); CKs (cytokinins), GAs (gibberellins), IAA (indole-3-acetic acid), Hex (hexoses), JA (jasmonic acid) and Suc (sucrose).

3. Strategies to improve abiotic stress tolerance in horticultural crop production

Despite the continued efforts of breeders and researchers to mitigate the water scarcity problem in crop production by obtaining or propagating varieties more tolerant to drought or other forms of abiotic stress, the solution does not seem close or simple (Mickelbart et al., 2015). With climate change, the effects of environmental stress are becoming more pronounced, which is the reason why studying new agronomic strategies against abiotic stress in crop management is gaining more and more interest. Currently, conservation agriculture principles underscore the importance of soil water conservation (Dumanski et al., 2006). Therefore, large frameworks of strategies are being used to optimize soil water content, particularly in arid and semiarid areas, by minimizing the amount of water evaporated from the soils (Grum et al., 2017). Among the different strategies that have been developed in order to cope with water scarcity in agricultural systems, this thesis is focused mainly on the use of new hydromulching formulations along with their interaction with arbuscular mycorrhizal fungi (AMF) as sustainable techniques to improve horticultural crop production. The following sections describe the effects of the use of these strategies, (hydro)mulching and AMF, on crop production as well as on the agroecosystem.

4. The use of mulching in crop production

It is well known that there is an imperative need to improve water use efficiency and sustainability in crop production, which is a pursued worldwide objective. Mulching is a management practice to save water in agricultural production systems (Biswas et al., 2016). Particularly, its use is very important in horticultural crops (López-Marín et al., 2012a). The English word mulch derives from the middle English word "molsh", which means soft or beginning to decay (Jacks et al., 1955), and is defined as materials, organic or in-organic, which are applied on the soil surface (Chalker-Scott, 1962). Mulches are thus a protective layer, usually made of organic matter, such as leaves or straw, placed on the soil surface around plants to prevent soil water evaporation, weed proliferation, and nutrient losses (Barche et al., 2015). Besides, mulching is defined as a cropping practice that entails placing organic or synthetic materials on the soil close to plants to manipulate the crop-growing environment due to its effects on crop efficiency and product quality by retaining soil moisture, protecting plant roots from extreme temperatures, reducing soil evaporation, suppressing weed growth via shading, and improving soil physicochemical characteristics (Chakraborty et al., 2008; Kasirajan

& Ngouajio, 2012; Lamont, 2005). Recently, the review carried out by Kader et al. (2019) pointed out a wide range of benefits of the use of mulching in agriculture. From a soil environmental approach, improves soil water conservation, reduces water evaporation, increases water holding capacity, helps pest and weed control, increases the nutritional status of the soil, and regulates soil temperature. From an economic perspective, improves crop yield, increases fruit quality, augments the efficiency in the use of water, and advances the harvest period.

4.1 Types of mulching

Mulches are generally classified according to their inorganic and organic origin (Figure 6). The inorganic mulching materials embrace polyethylene plastic films, which are the most widely used throughout the world, and synthetic polymers (Kyrikou & Briassoulis, 2007). The organic mulches are derived from animal and plant residues such as agricultural wastes (straw, stalks, saw dust), grasses and cover crops (living mulches), processing residues (rice hulls), and animal manures (Rathore et al., 1998). There exist several new types of organic and inorganic mulching materials as biodegradable and photodegradable plastic films, as well as sprayable and biodegradable polymer films for easy application and versatility (Adhikari et al., 2016; Yang et al., 2015).

4.1.1 Inorganic mulches

The most commonly used inorganic mulches throughout the world are polyethylene plastic films, which are petroleum-based products (Kader et al., 2017). Linear low-density polyethylene (LLDPE) is a thermoplastic made from the monomer ethylene and is one of the most widely used film plastic materials in agriculture due to its easiness of processing, excellent physical and chemical resistance, high durability, and flexibility (Zribi, 2013). Importantly, due to its characteristics, plastic films produce a relatively impermeable barrier to the flow of water vapor on the soil surface which increase soil water availability for the plants. Black plastic mulch is the industry standard (Tarara, 2000), but there exist also other colors with different optical properties (Reviewed by Kasirajan & Ngouajio, 2012). These differences in optical characteristics influence how plastic mulch modifies the microclimate around the crop (Gordon et al., 2006; Tarara, 2000). Despite the extensive benefits offered by the use of plastic mulches to the crop, the exacerbated use of plastic has supposed a serious environmental problem mainly due to its low degradation, its permanence in the field, and the potential risk of soil pollution. Furthermore, the cost of removing plastic debris is very high (Moreno et al., 2013), so the use of biodegradable mulch materials might be an alternative solution.

4.1.2 Organic mulches

Organic mulches generally come from crop residues that remain in the field after harvest, or from a wide variety of other products generally derived from plant debris. The most common material used for organic mulching is straw, but other frequent organic mulches used include paper mulches, which are made of wrapping paper, waxed or treated, providing a cheaper alternative to straw (Haapala et al., 2014). Wood waste represents another source of organic material for mulching.

Figure 6. Lettuce cultivation under (a) classical plastic films and under (b) straw mulch.

4.2 Benefits of mulching

The mulching technique establishes a linkage between soil and agrometeorology which can modify the crop-growing environment (Kader et al., 2019). The main environmental impact of mulching is produced on the microclimate soil–crop–air environment, since mulching modifies both soil and air temperatures, water evaporation rate from the soil, and the gas exchange among soil and air. All these changes in the crop environment have agronomic consequences. The soil temperature rise enhances crop development, whilst soil cooling produces the reverse result. Temperature increases in the air around the crop intensify the growth of the shoot, and therefore, the leaf area and crop yield (Dahiya et al., 2007). Moreover, limiting the light reaching the soil reduces weed growth, thus minimizing external competition for the crop (Gangaiah et al., 2019).

Mulches are proven to reduce water evaporation from the soil and increase soil water availability, avoiding hydric stress in plants and providing more water for roots (Kannan, 2020). Mulching significantly increases soil moisture in the topsoil compared to bare soil (Zhang et al., 2008). The use of polyethylene plastic mulch achieves the greatest effects on the water economy since its great impermeability prevents evaporation from the soil surface, improving the water available to the crop, which benefits from a more constant and regular supply (Jia et al., 2006; Liu et al., 2009; Maurya & Lal, 1981). Plastic mulching has been considered a potential water conservation practice in contemporary agriculture and is currently used in all types of edaphoclimatic areas due to its benefits to the agroecosystem (Kader et al., 2019). Between the numerous effects of plastic mulching on crop development, plastic mulches underscore to be a very effective technique for reducing soil evaporation, thus improving crop water use efficiency (Almeida et al., 2015) and minimizing salt build-up in the root zone (Dong et al., 2009; Yuan et al., 2009).

Organic mulches based on crop residues such as straw or bark also reduce evaporation by limiting the amount of radiant energy absorbed and minimizing airflow at the ground surface. The study of Peng et al. (2015) showed that straw mulches dramatically reduced evaporation rates and maintained higher soil moisture than bare soil. Dahiya et al. (2007) observed that plant residues reduced soil evaporation by an average value of 0.39 mm day^{-1} compared to the controls. However, Mellouli et al. (2000) concluded that the reduction in evaporation with organic mulches decreases with time. The effectiveness of mulches, both organic and inorganic, in preventing the evaporation of water from the soil has been well documented by various works. Leskovar et al. (2013), Ozer (2017), and Zhang et al. (2017) showed its effectiveness in horticultural crops, while Martin-Closas et al. (2016) presented the advantages of using mulching in tree crops. The mulching technique also affects soil structure. The structure of mulched soils remains in a better state than that of the bare soils due to the mulching protection against atmospheric agents. The beneficial effect of mulch on the soil structure is mainly a consequence of the buffering of the kinetic energy of raindrops, which reduces the soil physical dispersion and maintains the rate of soil water infiltration (Yan et al., 2011). Moreover, the increase in soil temperature and soil moisture favours soil mineralization, which generates a greater availability of nutrients for plants, including nitrogen, and soil organic carbon (SOC) (Irshad et al., 2022). In contrast to plastic films, organic mulches have a greater effect on the activity of soil microfauna and the proliferation of roots, which decreases soil compaction due to the aggregation of fine clay particles (Jordán et al., 2010). According to Sinkeviciene et al. (2009), the application of mulch based on straw on the soil surface increases the content of organic matter characterized by a high humification index. Webber et al. (2022) confirm these results, finding out that soil mulching with wheat straw generates more favourable habitats for earthworms, insects, and microorganisms, which contributes to reducing the bulk density of the soil compared to soil mulching with polyethylene. Soil mulched with organic material has a relevant effect on the increase of soil porosity, which allows good soil aeration and greater development of the root system (Luo et al., 2016), leading to better absorption of water and nutrients from the soil. Mulching also affects soil respiration due to its effects on soil moisture and fertility (Liu et al., 2009). Soil respiration can be defined as the amount of CO_2 released from the soil due to the decomposition of soil organic matter (SOM) by soil microbes and from the respiration of plant roots and soil biota (Zhang et al., 2015). It is an important indicator of soil health because it indicates the level of microbial activity, SOM content, and its decomposition. This phenomenon is the result of the production of CO_2, linked to the decomposition of organic materials in the soil. Mulching has an important effect on soil erosion as well. Reduced erosion also diminishes nutrient and fertilizer losses (Reviewed by Jordán et al., 2010). The study of Wakindiki & Danga (2011) concludes that the application of wheat straw mulch reduced the losses of NO_3^-, N, P, and K, in the soil. According to the study of Neilsen et al. (2007), paper shreds and alfalfa straw were used as mulches and they were able to increase the availability of soil minerals and enhanced microbial activity and root development, which positively affected growth. As a consequence of the short-term effects on soil conditions, plastic films afford an increase in product yield and product quality, as well as higher economic value for farmers (López-López et al., 2015). The effects of mulch in crop production depend on both the geographical area as well as the type of crop. In locations where direct solar radiation is predominant (e.g. Mediterranean basin), the mulch effects are considerably greater than where diffuse radiation is predominant (e.g. central Europe). Additionally, several cultural practices, such as soil preparation, crop season, irrigation system, and/or plant density, also could contribute to the mulch performance with the corresponding crop effects (Kasirajan & Ngouajio, 2012). The interaction between the use of mulching in the agroecosystem and climatic environment is illustrated in Figure 7, which summarizes the effects of the use of mulching in the crop environment.

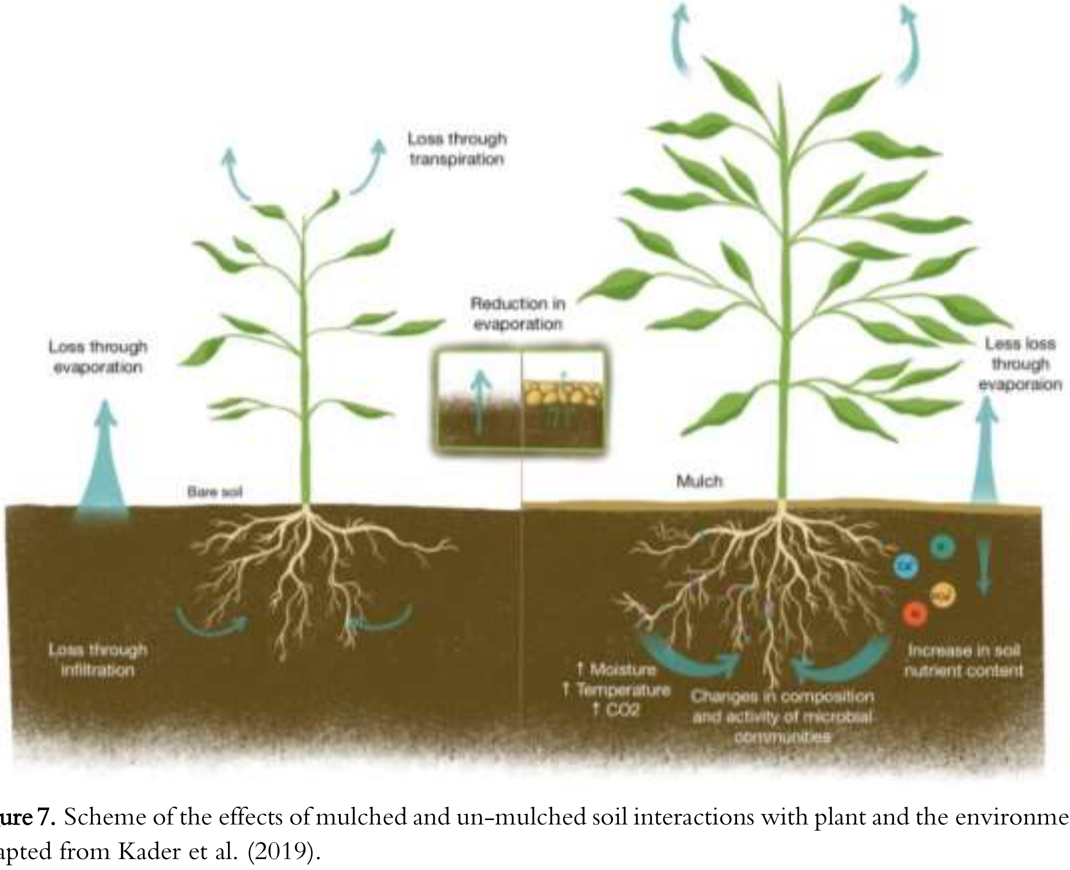

Figure 7. Scheme of the effects of mulched and un-mulched soil interactions with plant and the environment. Adapted from Kader et al. (2019).

4.3 Environmental problems derived from plastic mulch

The extended use of plastic in agriculture, called plasticulture, has increased sharply throughout the world since 2000 (Kyrikou & Briassoulis, 2007). Plastic films have been used since the 60s to enhance and improve horticultural production (Lamont, 2005). The use of plastic mulching in agriculture is getting popular all over the world, and its use is increasing day by day. According to the European association of packaging plastics products for agriculture, Europe has destinated around 1,300,000 t of plastics for agriculture in 2015. Spain is the most important European country in the manufacture and consumption of films, using more than 90,000 t, followed by Italy, Germany, and France (APE Europe, 2022). The use of black plastic film mulch is mainly due to its economical availability in comparison to other mulches (Ngouajio & McGiffen, 2002). Importantly, plastic mulches are very supportive in horticultural crop production mainly in dry and hot regions of the world, such as the Mediterranean basin.

In general, the use of mulches in agriculture has a series of technical-environmental advantages, but it implies an increase in production costs, mainly due to the cost of transport, installation, and handling of the mulches, as well as their subsequent management after the crop cycle. However, it is estimated that around two million tons of plastics are used in protected cultivation techniques in Europe for agricultural applications (APE Europe, 2022). This implies the accumulation of plastic wastes and the associated environmental impact, since only a small percentage of agricultural plastic wastes are currently recycled (López-Marin et al., 2012b). Despite polyethylene plastic films are not biodegradable, these materials undergo a photodegradation process during their exposure in the field, producing a reduction in film performance and their fragmentation into small pieces which remain on the soil and contaminate the environment (Schettini et al., 2011; Vox et al., 2007). The long permanence of plastic waste in the environment produces a huge and unmanageable plastic accumulation and becomes seriously harmful to the agroecosystem and human health (Ingman et al., 2015). All these reasons have led to a great interest in sustainable alternatives to plastic mulching.

4.4 Alternatives to plastic mulch: biodegradable films and hydromulches

The current food demand associated with the increase in world population has led to a change in the agricultural production systems, becoming imperative to use more environmentally sustainable techniques that allow food production as well as good quality products to sustain in the international market. Besides using crop varieties with high-yielding properties and good agricultural practices, it is necessary to implement sustainable methods for optimal yield and quality enhancement. In the case of mulching, the application of new mulching materials that come from natural resources has currently become one of the most interesting agrotechnology of sustainable crop production.

4.4.1 Biodegradable films

Different strategies have been proposed to reduce plastic pollution, being the most effective ones those incentivizing the use of degradable films. Depending on its degradation process there exist different degradable films. The terms "oxo", "hydro", "chemo", "photo" degradable films describe abiotic mechanisms of degradation. On the contrary, "bio" degradable film indicates the biological process by which soil microorganisms present in the agroecosystem use carbon substrates as food for their life processes. The most common alternative to plastic films are biodegradable films. These biodegradable mulches are classified according to their source of origin in fossil-based plastics, which are polymers made from petrochemicals such as polycaprolactone (PCL), and bio-based plastics, which are composed of biopolymers formed from renewable resources and are basically composed of polysaccharides such as cellulose and starch. The most usual starch film is based mainly on corn, potato, and rice crops, and among their intrinsic characteristics, they offer low permeability and are mineralized into harmless products (CO_2, methane, water, and biomass) by soil microbiota (Chandra & Rustgi, 1998; Moreno & Moreno, 2008; Scarascia-Mugnozza et al., 2004).

Nevertheless, the biodegradation process depends on the geographical area and the edaphoclimatic conditions, since favourable circumstances of temperature, light, soil moisture, oxygen, etc. must be given for the biodegradation process to occur in a relatively short period of time (Kyrikou & Briassoulis, 2007). Indeed, the edaphoclimatic characteristics of the arid and semi-arid zones, such as the Mediterranean basin, which present low SOM and low soil moisture, limit the action of soil microbiota thus greatly hampering the natural degradation of these materials, resulting in soil contamination. Recently, Sintim et al. (2019) pointed out that there is limited information on the possible repercussions of biodegradable films on soil health. Despite a great deal of research about biodegradable plastics, it is important to consider that, apart from the problem of their degradation in arid and semi-arid areas, there is a serious economic limitation to using them due to the elevated cost of these films (Kasirajan & Ngouajio, 2012). Therefore, research efforts are currently focused on the study of sustainable alternatives to plastic films in these climatic areas.

4.4.2 Hydromulches

Considering the previous statements, it is not surprising that the use of organic mulches has gained interest again, although organic by-products from crop wastes have been used as mulches for many years. Hydromulch is a new organic mulching alternative that is currently under study and is based on paper pulp in combination with natural additives. Recently, the possibility of reusing paper as mulch was seriously reconsidered (Haapala et al., 2014). Paper is an organic material employed as mulching before the plastics era (Verdú et al., 2020), and due to its properties, it was used in formulations that involved coated paper with several materials (Shogren, 2000). Recovered paper is used as a raw material to make recycled paper and other paper products. One possible application for recycling recovered paper and cardboard is to produce biodegradable mulch materials that are applied as a sludge until they later solidify on the soil surface, maintaining their integrity throughout the growing season and producing the beneficial effects of a mulch cover (Warnick et al., 2006). "Hydromulch" can be defined as a mixture of water with some kind of lignocellulosic material, in combination with other suitable additives, which is applied as a paste, rather than as a film, that covers the soil surface. Hydromulch blends dry in a general interval of 12 h from its application and ends as a layer on the top of the soil surface.

Currently, it is necessary to enforce the principles of sustainable agriculture and development, which refer to the need to minimize the degradation of agricultural land, while maximizing production. In this sense, one of the new objectives of many governments is the transition to a more circular economic model with products, processes, and business models designed to maximize the value or utility of resources, while at the same time reducing adverse impacts on health and the environment (European Union, 2017). Therefore, the use of available and low-cost organic agricultural residues has been proposed to compose biodegradable mulch materials (França et al., 2019; Liu & Gao, 2018). Also, hydromulch would provide an outlet for recovered paper. Agricultural waste materials, such as rice hulls, the substrate used for mushroom cultivation, and wheat straw have the intrinsic potential to be used as hydrocompost, which would be an interesting option for the production of horticultural crops.

Originally, the use of hydromulches was extendedly used in the rehabilitation of degraded lands, to mitigate the risk of runoff, and soil erosion, and for burned soil reforestation (Warnick et al., 2006). However, studies about the beneficial effects of hydromulch in the agroecosystem are very scarce. Among the potential benefits of the use of hydromulch may be as a long-term biodegradable mulch that remains intact and effective in the course of the growing season. Due to its organic origin, this type of mulching can provide a source of nutrients, dissolved organic carbon, and an extra microbiome that could have a positive influence on crop development. Unlike plastic films, the use of hydromulches is suitable for tillage practices with applicability in sustainable conventional production systems. Indeed, one of the main objectives of this thesis is to evaluate the effects of the use of hydromulch on the development of several horticultural species as well as their effect on soil characteristics.

5. Arbuscular mycorrhiza fungi as a strategy to increase crop productivity

Arbuscular mycorrhizal fungi (AMF) are soil microorganisms that form a symbiosis with 80% of terrestrial plants (Weng et al., 2022; Begum et al., 2019) generating arbuscules, vesicles, and hyphae within the cortical cells of the plants they colonize (Figure 8). Its distribution is also wide, since they are found in all ecosystems and soils, and can be very heterogeneous in the same place in terms of variety and quantity, which is an important requirement to obtain the maximum benefit from the association (Douds & Millner, 1999). These root-fungus associations play essential roles in the absorption of mineral elements in terrestrial ecosystems. The two partners mutually exchange elements necessary for their proper development: fungi (heterotrophs organism) provide mineral elements to the plant (autotroph organism) in exchange for carbon molecules from the photosynthesis. The symbiotic association between the fungus and the plant acts as a complement of the root of the plant in the uptake of nutrients, especially in the absorption of phosphorous (Balliu et al., 2015; Smith et al., 2011), increase in tolerance to abiotic stress conditions (Raklami et al., 2019), improvement of soil quality, better N_2 fixation, and higher plant diversity and productivity in a given ecosystem (Liu et al., 2019; van der Heijden et al., 2006). Nutrient mobilization can occur through an enzymatic pathway that allows the fungus to use organic nitrogen and phosphorus, or through the release of organic acids mobilizing calcium, magnesium, and potassium, among others (Landeweert et al., 2001).

5.1 AMF-plant symbiosis

The establishment of symbiosis begins with the colonization of a compatible root by hyphae from a propagule. Following this fungus asymbiotic stage, the first stage of mycorrhizal colonization is the formation of an appressorium on the surface of the root from which the fungus can penetrate the epidermis and root cortex to form the intracellular structures, vesicles and arbuscules (Requena et al., 2007). During the presymbiotic stage, there exists a chemical “dialogue” between the fungus and the compatible root which allows the meeting of the two partners. Root exudates from the host plant induce the germination of spores, growth, and branching of the hypha (Buee et al., 2000), which increases the chances of the fungus coming into contact with the root. According to the review of Bonfante & Genre (2010), after the formation of the appressorium, an opening of the root epidermis allows the passage of the hypha which can then progress through the cells of the outer cortex. After this intracellular passage in the superficial layers of the cortex, the fungus comes out of the cells and passes through the apoplast at the level of the inner cortex and can proliferate along the axis lengthwise of the root. The hypha then forms branches that penetrate the cells of the inner cortex and form arbuscules. Thus, the fungus-cell interface root is always composed of the membranes of both partners, generating between the two a peri-arbuscular space where the exchanged elements can be transferred or accumulated. The arbuscules constitute the place of exchanges of nutrients between the plant and the fungus. Apart from the arbuscules, we can find other fungal structures such as vesicles, spores and endogenous hyphae (Figure 8). The AMF-plant symbiosis increases the supply of nutrients, mainly phosphorus, which is translocated to the plant in a higher percentage with respect to other nutrients (Smith et al., 2011).

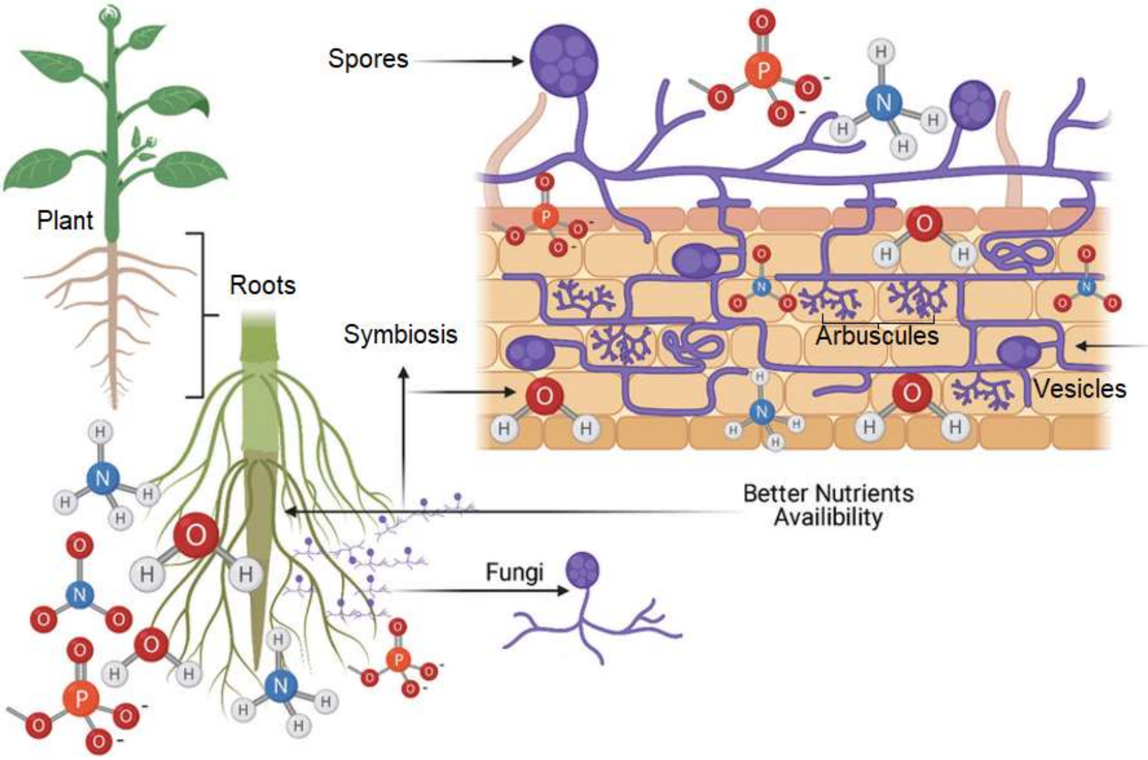

Figure 8. Scheme of the root colonization process and nutrient uptake by AM fungi. Adapted from Wahid et al. (2022).

5.2 Effects of AMF on crop production

One of the main objectives of sustainable agriculture is the reduction of the use of fertilizers due to their negative effects on the environment (Giang et al., 2015). An alternative to the use of chemical fertilizers is biological fertilization, which provides plants with their nutritional requirements through the various functions of some microorganisms such as AMF. In agriculture, the use of AMF has great biotechnological potential since they facilitate the availability of nutrients for plants besides other benefits (Raklami et al., 2019). The importance of AMF in agriculture lies in their extensive ERM. Mycorrhized plants present advantages in terms of the absorption of nutrients with low soil mobility, since the external mycelium extends to a greater distance in the soil than the root hairs of the non-mycorrhizal plants (Begum et al., 2019). Mycorrhization has been found effective in improving the growth and productivity of host plants. There are numerous reports on the significant stimulation of mycorrhized plants and the increase in crop yield in several agronomic species, such as chickpeas (Oliveira et al., 2017), maize (Selvakumar et al., 2017), and tomato (Balliu et al., 2015), among others. Indeed, the well-known effect of AMF on phosphorous absorption and the acquisition of other nutrients is normally linked with a considerable increase in biomass (Azcón-Aguilar & Barea, 1997). Furthermore, several authors have stated that AMF colonization affects a broad range of root system morphological parameters, with greater root branching as the most common effect. It has been stated that changes in the hormonal balance may explain these impacts on plant growth, particularly under abiotic stress (Khalloufi et al., 2017). Among the main effects of AMF inoculation in horticultural crops, the reviews of Barea et al. (1993) and Verzeaux et al. (2017) pointed out beneficial effects on plants, such as the increase in plant growth, reduction in phosphorous requirement, an increase of survival rate and plant development, higher resistance to fungal root pathogens, improve tolerance to abiotic stresses and greater yield (Figure 9).

A very interesting characteristic of AMF is its protective effect on the host plant to confront abiotic or biotic stresses. Indeed, drought is one of the main abiotic factors encountered by plants and the association with mycorrhizae helps to reduce the stress symptoms. Furthermore, it has been reported the AMF effect on mitigating nutrient deficiencies, improving drought tolerance, exceeding the detrimental effects of salinity, and enhancing plant tolerance to polluted soils with polyaromatic hydrocarbons and/or heavy metals (Calonne-Salmon et al., 2018; le Pioufle et al., 2019; Piouffle et al., 2021). The mechanisms that explain the protective action to environmental stresses of AMF-plant symbiosis include plant nutrition enhancement, damage compensation, induction of changes in the root system morphology, activation of plant defense mechanisms, regulation of plant hormone balance, and adjustment of secondary metabolism (Reviewed by Kaur & Suseela, 2020).

Rhizophagus irregularis is one of the most studied AMF and several studies have shown that can establish symbiosis with a wide variety of agricultural plants, improving growth (Tekaya et al., 2017), quality (Yang et al., 2020) and crop yield. (Navarro & Morte, 2019). *R. irregularis* captures phosphorous directly from the soil or by hydrolysis of complex forms of soil organic phosphates, it polymerizes into polyphosphate and it is translocated through the extraradical mycelium (ERM) to the intraradical mycelium (IRM), finally releasing phosphorous to the periarbuscular space where it is translocated to the plant by specific transporters (Walder et al., 2016). The main role of mycorrhizae is to increase the efficiency of the plant to absorb nutrients, solubilizing them or increasing the extension of the root system through external hyphae that can explore inaccessible areas to the root (Liu et al., 2019). In addition to increasing phosphorous uptake, mycorrhizae can absorb and transfer significant amounts of mineral nitrogen in the form of nitrate and/or ammonium to their host plant (Verzeaux et al., 2017). There are also indications of the transport of organic nitrogen, especially in the form of amino acids. The availability of this nutrient limits plant development and, depending on soil conditions, nitrogen transference by mycorrhizal fungi can represent an important route in its uptake by the plant. Intracellular ammonia is transported in the form of arginine from the ERM to the IRM, ammonium is again catabolized and released into the periarbuscular space to transfer to the host plant (Tian et al., 2010). *R. irregularis* mainly provides nitrogen and phosphorous to the host plant in exchange for carbohydrates, mainly in the form of glucose (Ait Lahmidi et al., 2016) and lipids in the form of β-monoacylglycerides (Jiang et al., 2017). However, the use of the AMF *R. irregularis* as a large-scale inoculum is still not widely used due to some limitations such as the difficulties to obtain large quantities of inoculum and high competitiveness with native soil AMF. Additionally, other factors must be taken into account, such as their relationship with microbial communities (Hestrin et al., 2019; Idbella et al., 2021; Rozmoš et al., 2022) and soil fungi (Renaut et al., 2020), and the possible effects on AMF of physical soil disturbance and fertilization practices (Peyret-Guzzon et al., 2016).

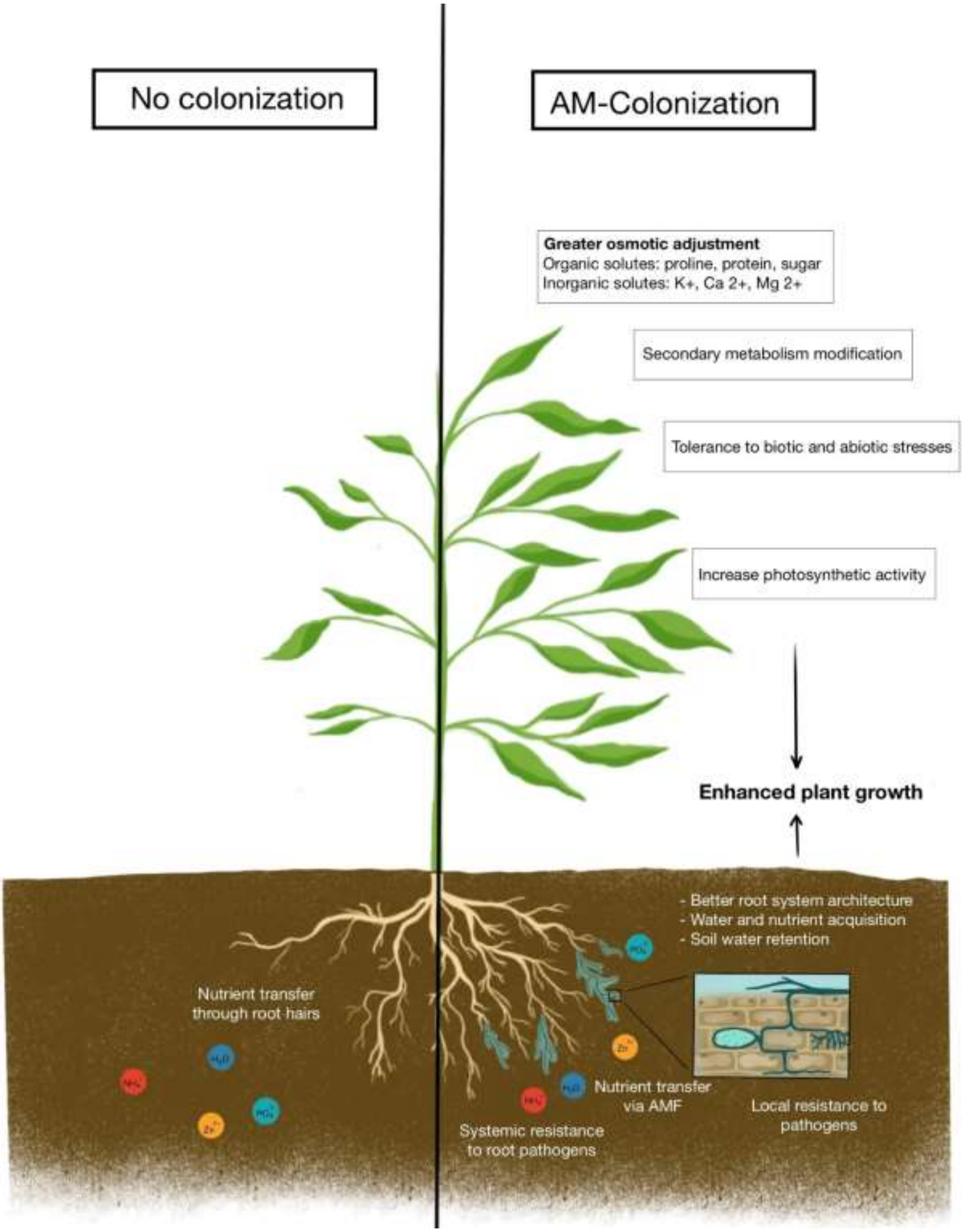

Figure 9. Scheme of the effects of AMF in plant development. Based on Jacott et al. (2017).

6. Importance of artichoke and escarole crops

The horticultural crops on which this thesis is focused are artichoke (*Cynara cardunculus*. var. *scolymus* L. Fiori) and escarole (*Cichorium endivia* L.). Each of these two crop species represents different cultivation cycles and management. In the case of the artichoke, it is a long-cycle crop, with plantations during the summer months and harvesting during the spring period, which can last under culture for two years. Regarding escarole, it is a short short-cycle crop that can be done both in the spring months as well as in the colder months, and its harvest is done approximately 90 days after transplanting. One of the aims of this thesis is to evaluate the repercussion of the use of more sustainable mulching techniques (hydromulching) in short-cycle crops (escarole) and long-cycle crops (artichoke). In the following sections, the worldwide importance of the cultivation of both crop species has been described.

6.1 Artichoke relevance in crop production

Artichoke (*Cynara cardunculus* L. var. *scolymus* Fiori) belongs to the Asteraceae family. It is a diploid, semi-perennial, cross-pollinated plant. The plant is silvery green in color and grows to an average height of 1.0–1.5 m covering an area 1.5– 2.0 m in diameter. However, some of the current cultivars have higher sizes (Bianco, 1990; Pignone & Sonnante, 2004). After the appearance of the leaves, and coinciding with a morphological change of the same to slightly more entire edges, the erect and thick stem, longitudinally grooved, elongates and branches, reaching a height of 1.5 m. At the end of the stem, the inflorescence growth in chapters, which constitute the edible part when they are tender and closed. The leaves are elongated, large, fissured, grayish-greenish on the upper side, and hairy on the lower side, with a prominent vein. The leaves that surround the head make up the bracts. It must be consumed before the flower develops since in that case the receptacle hardens and forms thorns (López, 2000). Artichoke is a highly valued food in the Mediterranean diet due to its healthy nutritional properties. Its high humidity, low caloric content, practical absence of fat, and its fibrous nature have made it one of the dietary foods of excellence. Another important characteristic is the high content of minerals, especially potassium, sodium, magnesium, phosphorus, and calcium, which are essential for health since they participate in numerous functions such as the regulation of fluids in the body, blood pressure control, functioning of the intestine, muscles, and nerves, etc. (de Falco et al., 2015). According to Grabowska et al. (2018), artichoke cultivation is viable in the regions of the world where the climate has cool summers and mild winters. Most of the world's production is concentrated in the Mediterranean basin. This trend is not only due to the climatic conditions but also to the ancestral origins of this crop. The artichoke is very demanding in water; and when water is limited smaller chapters are obtained, which present less resistance to transport. In contrast, with optimal irrigation, the heads are juicier, larger, and resistant to transport. Artichokes can be propagated from seeds or vegetatively. Originally, the artichokes have been cultivated by vegetative multiplication, while currently their cultivation by seed is mainly due to the large amount of crop failure that originates from vegetative multiplication (Sonnante et al., 2007). In this thesis, we have assayed a new seed variety called "Symphony" (Nunhens-BASF).

Figure 10 shows the world production of artichoke in the year 2020, most production is concentrated in the Mediterranean areas of Europe and Africa according to the data obtained from the Food and Agriculture Organization Corporate Statistical Database (FAOSTAT; FAO, 2022). The production of Italy stands out followed by Egypt and Spain, together representing around 60% of the total world production. Spain is the third greatest artichoke producer in the world. Artichoke production in Spain was around 196 thousand tons in 2020. The main artichoke exporter in the world is Spain, followed by France, Egypt, and Italy. Other large artichoke producers are Peru, Argentina, China, France, Algeria, United States of America, Morocco, Chile, and Turkey.

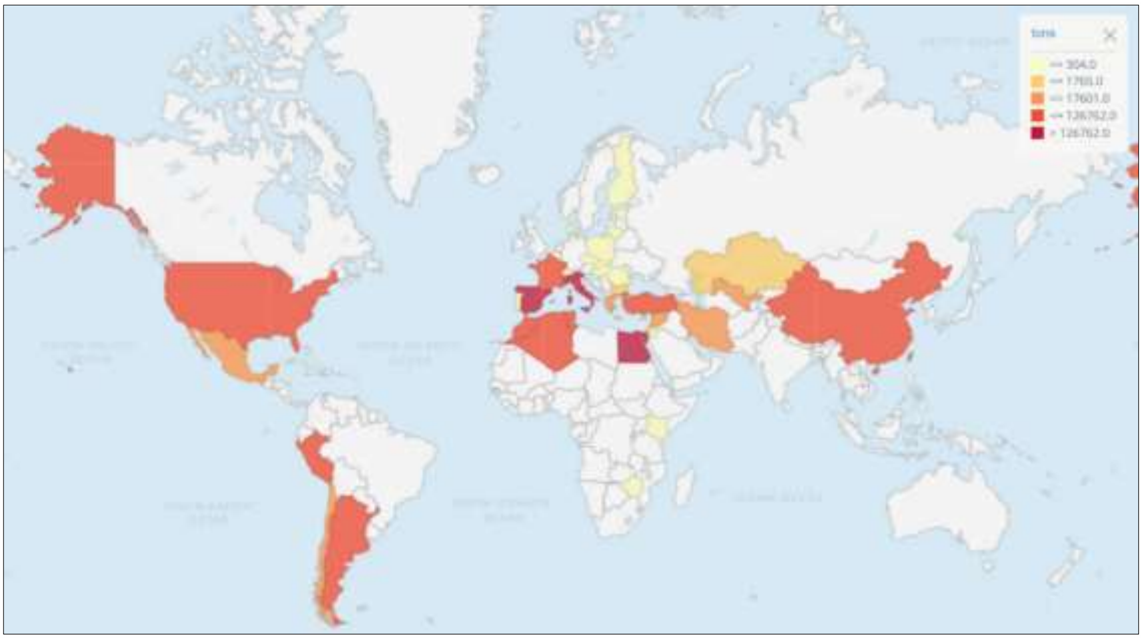

Figure 10. A choropleth map showing countries by artichoke production in tonnes, based on 2020 data from Food and Agriculture Organization Corporate Statistical Database (FAO, 2022).

6.2 Escarole relevance in crop production

Escarole (*Cichorium endivia* L) belongs to the Asteraceae family and its origin seems to be in East India. There are two varietal groups based on the shape of their leaves: The Crispas varieties, with divided leaves and jagged edges, and the Latifolia varieties, with wide, wavy leaves and slightly jagged edges. The commercial varieties "Brillantes" and "Bekele", chosen for this thesis, belong to the Latifolia group. The plant has rosette leaves and does not form a heart shape. It is considered an appetite-stimulating plant due to its slightly bitter taste owing to the presence of lactucin and lactucopyrin, (Maroto, 2000). Escarole, along with lettuce, is one of the most used vegetables in 'IV range' salads. In addition to its beneficial effects on health, attributed to its high content of antioxidants and fiber, it has good adaptability to the cold chain, not having serious oxidation or microbiological problems (Llorach et al., 2008; Rico et al., 2007). In recent years, the consumption of this type of product has increased due to changes in consumer preferences (Baslam et al., 2013). According to the FAO data (FAO, 2022), the world production of escarole and lettuce in the year 2020 was mainly concentrated in Asia, followed by Europe and America (Figure 11). The production is centered in China, India, and United States of America, which represent around 70% of the total world production. Among the other important producers are the Mediterranean countries Spain and Italy, followed by Japan, Mexico, Belgium, Turkey, and France. In Spain, lettuce and escarole can be grown all year round in all systems (de Rada et al., 2010; Romero-Gámez et al., 2014). Spain produced 969 thousand tons of lettuce and escarole in 2020 (FAO, 2022).

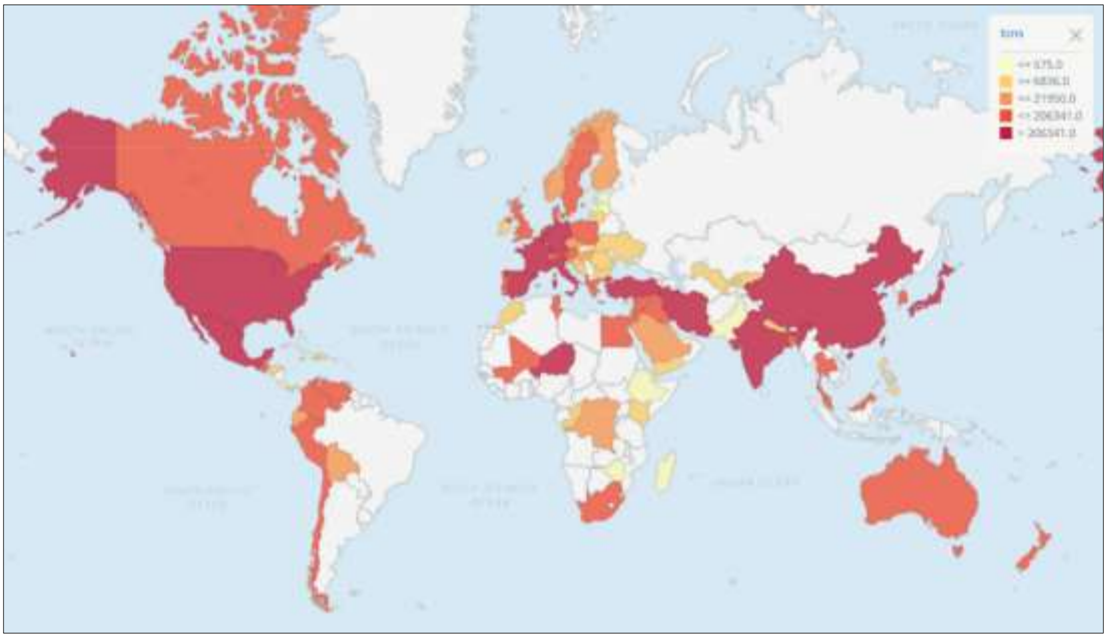

Figure 11. A choropleth map showing countries by lettuce and escarole production in tons, based on 2020 data from the Food and Agriculture Organization Corporate Statistical Database (FAO, 2022).

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CHAPTER II

The use of hydromulching increases yield and quality of artichoke (*Cynara cardunculus* var. *scolymus*) by improving soil physicochemical and biological properties

The use of hydromulching increases yield and quality of artichoke (Cynara cardunculus var. *scolymus*) by improving soil physicochemical and biological properties

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Abstract

We hypothesized that organic liquid mulching formulations (hydromulching) can be used in sustainable agricultural production through their direct influence on physicochemical and biological soil properties to improve crop yield and quality. Artichoke plants were grown using three hydromulching treatments, rice hulls (RH), wheat straw (WS), and mushroom substrate (MS), and two control treatments, soil covered with polyethylene (PE) and bare soil (BS). Soil quality-related parameters and artichoke head physical and chemical properties were measured for each treatment. Soil organic matter and carbon increased in all hydromulching treatments, especially with the RH cover. Hydromulched soils showed higher soil moisture, especially in WS-covered soil, and an improvement of the soil ions K^+, P, Cu^{2+}, Mn^{2+}, and Zn^{2+}, particularly in MS-covered soil. The mulched treatments produced the highest crop yield, while the lowest yield was obtained in bare soil. Importantly, plant water relationships were superior in all mulching treatments giving rise to the improvement of crop yield and the most important physical quality parameters in artichoke, namely color, firmness, and size. Furthermore, the content of some important nutrient elements in artichoke (K^+ and N) were greater in RH and MS treatments, while sugar concentrations were higher in bare soil with respect to the other treatments. Our results demonstrate that hydromulching is a sustainable alternative to plastic mulching that regulates plant hydric status and soil moisture and fertility, thus improving yield and physical quality characteristics of artichoke heads, and maintaining their chemical and nutritional quality over bare soil.

Keywords: Mulching; Sustainable agriculture; Plant water relations; Soil fertility; Artichoke quality.

CHAPTER III

Hydromulching enhances the growth of artichoke (*Cynara cardunculus* var. *scolymus*) plants subjected to drought stress through hormonal regulation of source-sink relationship

Hydromulching enhances the growth of artichoke (Cynara cardunculus var. *scolymus*) plants subjected to drought stress through hormonal regulation of source–sink relationships

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Abstract

Mulching the soil with organic-based formulations (hydromulching) is a sustainable alternative to plastic mulching that is here hypothesized to maintain crop production under drought stress by hormonal and metabolic regulation of source–sink relationships. To test this hypothesis, artichoke plants were grown on non-mulched soil and on soil mulched with polyethylene and three different organic mixtures and subjected to optimal and reduced irrigation regimes. Under drought stress, the growth parameters were higher in plants grown with the different mulching treatments compared to non-mulched plants, which was related to a higher photosynthetic rate and water-use efficiency. Importantly, mulching-associated growth improvement under stress was explained by higher sucrolytic activity in the leaves that was accompanied by a decline in the active cytokinins. Besides this, salicylic acid decreased in the leaves, and abscisic acid and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid were impaired in the artichoke heads, which is associated with better regulation of photoassimilate partitioning. Taken together, these results help to explain the hydromulching-associated growth improvement of artichokes under water stress through the hormonal regulation of sucrose metabolism, which could be very useful in future breeding programs for drought tolerance.

Keywords: hydromulching; artichoke; water stress; source-sink relationships; sucrolytic activity; plant hormones

CHAPTER IV

The use of ecological hydromulching improves growth in escarole (*Cichorium endivia* L.) plants subjected to drought stress by fine-tuning cytokinins and abscisic acid balance

The use of ecological hydromulching improves growth in escarole (*Cichorium endivia* L.) plants subjected to drought stress by fine-tuning cytokinins and abscisic acid balance

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Abstract

Drought is considered as one of the major limiting factors to plant growth and productivity. Drought stress reduces stomatal conductance, affecting water relations and decreasing CO_2 assimilation rate and photosynthesis. Several strategies have been developed to alleviate the negative effects of drought in the agricultural industry. One of these strategies is the use of the mulching technology, which retains water in the soil surface. Knowing that hormones play a key role in plant growth and drought stress responses, we hypothesized that the use of a new ecological mulching technology called hydromulching would improve growth over bare soil under drought stress through changes in the hormonal balance. To test this hypothesis, escarole plants (Cichorium endivia L.) were grown in pots filled with coco fiber, non-covered (bare soil) or covered with polyethylene film (PE) and three types of hydromulches made up with recycled additives: wheat straw (WS), rice hulls (RH), and substrate used for mushroom cultivation (MS). Half of the plants were subjected to drought by reducing the volume of irrigation water to 70% of crop evapotranspiration. Despite drought stress impaired escarole growth-related parameters in all treatments, plants mulched with MS maintained significantly superior growth, due to improved plant water relations and photosynthetic function. This can be explained by an efficient interaction hydromulch/soil/plant in regulating the hormonal balance under water depletion. Indeed, the concentrations of the active cytokinins (CKs), trans-zeatin and isopentenyladenine, were higher in plants grown with MS treatment, associated with shoot growth-enhancing and photosynthetic rate maintenance under stress conditions. The concentrations of the stress-related hormone, abscisic acid (ABA), varied antagonistically to those of the active CKs. In this regard, ABA increased with drought but to a lower extent in MS plants thus regulating stomata opening, which, in crosstalk with the ethylene precursor 1-aminocyclopropane-1-carboxylic acid and salicylic acid, improved plant water relations. The results obtained demonstrate that hydromulching is an efficient and sustainable management strategy to ameliorate the drought effects on escarole plants through fine regulation of the CKs/ABA balance, which will be of utmost interest and applicability in the actual climate change scenario.

Keywords: escarole; hydromulching; drought stress; hormonal balance; cytokinins; abscisic acid

CHAPTER V

The Interaction between Hydromulching and Arbuscular Mycorrhiza Improves Escarole Growth and Productivity by Regulating Nutrient Uptake and Hormonal Balance

The interaction between hydromulching and arbuscular mycorrhiza improves escarole growth and productivity by regulating nutrient uptake and hormonal balance

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Abstract

The agriculture industry is frequently affected by various environmental stressors that reduce the efficiency of the plant in the use of the available resources, thus limiting its productivity. To improve water and nutrient use efficiency some strategies has been proposed, such the use of mulching techniques, or arbuscular mycorrhizal fungi (AMF) inoculation. To gain insights about the interaction between the use of hydromulch and AMF inoculation on plant growth and productivity, escarole plants (Cichorium endive, L.) were inoculated or not with the AMF *Rhizophagus irregularis* and grown under different soil cover treatments: ecological hydromulching based on substrate of mushroom cultivation (MS), low density black polyethylene (PE) and non-covered soil (BS, control treatment). Results revealed that AMF inoculation or the use of mulching alone, but especially their interaction, increased plant growth. The growth improvement observed in AMF-inoculated escarole plants grown under hydromulching conditions was mainly associated to the upgrading of nitrogen and phosphorous use efficiency through the regulation of the hormonal balance. Both hydromulching and AMF inoculation were found to increase the active gibberellins (GAs) and cytokinins (CKs), resulting in a positive correlation between these hormones and the growth-related parameters. In contrast, the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) and abscisic acid (ABA) decreased in AMF inoculated plants and especially in those grown with the hydromulching treatment. This study demonstrates that there exists a positive interaction between AMF and hydromulching which enhances the growth of escarole plant by improving nutrient use efficiency and hormonal balance.

Keywords: *Rhizophagus irregularis,* AMF, Cytokinins, Gibberellins, Escarole, Nutrient use efficiency

1. Introduction

Escarole (*Cichorium endivia* L.) is one of the most used vegetables in the preparation of ready-to-eat salads, with increasing interest worldwide for its characteristic crunchy texture and mildly bitter taste (Filippo D'Antuonno et al., 2016). In the Mediterranean area, escarole can be grown throughout the year in all crop systems (Romero-Gámez et al., 2014), and it is considered a very important crop whose production has increased during the last 10 years, reaching a total world production of 27.66 thousand tons in 2020 (FAOSTAT, 2022). In recent years, the concern about environmental sustainability has increased along with the growing demand for feed and food resources (Noya et al., 2018). In this context, current agricultural production practices have to embrace modern agricultural technologies in order to achieve food security for an increasing population. Recently, much attention has been paid to the use of different agronomic management strategies as the application of arbuscular mycorrhizal fungi (AMF), which are known to be involved in the improvement of plant growth and crop productivity (Poveda et al., 2019; van der Heijden et al., 2006). Several studies have shown a direct implication of mycorrhiza in terms of nutrient absorption and translocation due to the extra-radical mycelium that can effectively improve nutrient uptake, thereby improving plant growth and development (Li et al., 2016; Mitra et al., 2020). Indeed, Balliu et al. (2015) stated an increase in the leaf area, nitrogen, potassium, calcium, and phosphorous contents which enhanced plant growth rate in AMF-inoculated tomato plants. Other authors have shown the positive effect of AMF inoculation by improving mineral uptake, chlorophyll synthesis, and water use efficiency under salinity conditions (Begum et al., 2019). The growth boost due to mycorrhizal association can be explained by several mechanisms used by fungi under certain conditions (Bonfante & Genre, 2010). These include the increment of mineral uptake, mainly phosphorous, as well as the production of secondary metabolites as amino acids, vitamins, and hormones (Begum et al., 2019; Hashem et al., 2018; Kaur & Suseela, 2020; Mitra et al., 2020). Concerning phytohormones, it has been demonstrated their key role in the regulation of mycorrhizal symbioses (Gutjahr, 2014; Liao et al., 2018; Pozo et al., 2015). Various studies have shown the implication of gibberellins (GAs) in the arbuscule formation in plant root (Foo et al., 2013), while other early report suggested that AMF are involved in the upregulation of GAs in tomato plants (Khalloufi et al., 2017). Cytokinins (CKs) have been considered an important hormone class related to growth enhancement in horticultural crops (Gálvez et al., 2020; Romero-Muñoz et al., 2022). In this regard, it has been reported that a stronger increase in the shoot CK content were accompanied by elevated root CK levels associated with a positive plant growth response to AM symbiosis (Barker & Tagu, 2000; Shaul-Keinan et al., 2002). Cosme et al. (2016) and Martínez-Medina et al. (2011) have found that both shoot- and root-specific alterations of CK levels may be related to growth responses in inoculated tobacco and melon plants.

AMF have been also demonstrated to produce changes in the levels of other hormone classes. Some studies have reported significant changes in the concentrations of the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), and indoleacetic acid (IAA) in mycorrhizal plants (Jentschel et al., 2007; Martínez-Medina et al., 2011), depending on the AM fungus involved. Other studies informed that jasmonic acid (JA) content increased in the roots of mycorrhizal *Medicago truncatula* and soybean seedlings (Meixner et al., 2005; Stumpe et al., 2005). Regarding abscisic acid (ABA), the relation between ABA and growth of AMF plants has been so far unclear. Some authors have stated that mycorrhization increased ABA concentrations in the host plants (Miransari et al., 2014), while other authors have reported no effects of AMF inoculation on the ABA content (Martínez-Medina et al., 2011).

To date, a large number of studies have been done to investigate the effects of AMF on plant growth and productivity. Besides, traditional plastic mulching has been also demonstrated to be beneficial for plant productivity, increasing crop yield and quality (Iqbal et al., 2020; Liu et al., 2009; Sekara et al., 2019). Nevertheless, there is an urgent need to investigate new alternatives to the use of plastic in crops due to the environmental problems generated by its use (Kader et al., 2019). Recently, several authors have reported the benefits of the use of hydromulching, a new semiliquid soil cover formulation, which is based on different biological additives from crop residues (Claramunt et al., 2020; Verdú et al., 2020). Furthermore, it has been demonstrated the positive effect of the use of different types of hydromulches on plant yield in artichoke plants (López-Marín et al., 2021). Very recently, Romero-Muñoz et al. (Romero-Muñoz et al., 2022) have shown a direct implication of the hormonal balance regulation on the growth enhancement of hydromulched escarole plants under drought conditions.

To the best of our knowledge, no studies have been reported so far on the interaction of AMF with sustainable soil cover materials. Considering that both AMF inoculation and hydromulching have been reported to improve plant growth, we hypothesized that their interaction could have a synergic effect on plant productivity explained by the regulation of nutrient use efficiency and hormonal balance.

2. Material and Methods

2.1 Biological material and experimental design

The AM fungus *Rhizophagus irregularis* (Błaszk., Wubet, Renker and Buscot) C. Walker and A. Schüßler as ['irregulare'] was obtained from the collection of the Experimental Field station of Zaidín, Granada, Spain (EEZ 6). The AM fungal inoculum consisted of a mixture of rhizospheric soil from pot cultures (*Sorghum* spp.) that contained spores, hyphae, and mycorrhizal root fragments, with a potential infectivity of ~35 infective propagules per gram inoculum (Kohler et al., 2008). For the mycorrhizal (M) treatment, 10 gr of substrate was mixed with the AMF-inoculum substrate (coconut fiber/vermiculite, 1/2, w/w). The inoculum was placed adjacent to the root of the seedling. For the non-mycorrhized (NM) treatment, the substrate was prepared in the same final ratio (1:2) without AM fungal inoculum. Mycorrhizal (M) and non-mycorrhizal (NM) three weeks old escarole plants (*Cichorium endivia* L.) plants cv. Bekele were transplanted to 2.5-L containers. Two mulching treatments were installed on top of the substrate: the hydromulching treatment, based on a substrate used for mushroom cultivation (MS), and low-density black polyethylene (PE). The non-covered treatment (BS) was used as a control. The escaroles were irrigated regularly with Hoagland nutrient solution (Hoagland & Arnon, 1950). A modified Hoagland solution (i.e., 70% P-impoverished solution, referred to as Hoagland-P^{low} throughout the text) was used to irrigate the mycorrhized plants. The irrigation was performed by self-compensating drippers (2 L h^{-1}) and a fresh nutrient solution was applied to avoid nutrient imbalance. The amount of water used to irrigate the plants was adjusted every week, depending on the demand of the plants.

Plants were grown in a climate-controlled growth chamber under 16 h daylight period. The air temperature ranged from 22 to 25°C during the day. Relative humidity was maintained at 70±5% and the light intensity was approximately 200 μmol m^{-2} s^{-1}. Escarole plants were harvested 55 days after transplant. The experiment was designed as a factorial combination of AM fungal inoculation treatment (NM and M) and the use of different mulching treatments (BS, PE, and MS), which entails six combined treatments in total.

2.2 Plant Growth-Related Determinations

Plant growth-related parameters were recorded at the end of the experiment in 4 plants per mulching treatment in M and NM plants. Plants were washed with distilled water and separated into shoots and roots. The parameters recorded were shoot fresh weight (FW), root FW, leaf number, and leaf area (LA). Total fresh weight (TFW) was calculated as the sum of root and shoot FW. LA was quantified using a LI-COR leaf area meter (Model LI-3100C; LI-COR, Lincoln, NE, USA).

2.3 AM fungal root colonization

Roots were sampled and analyzed via ink staining (Walker, 2005). The roots were cut into small pieces and placed in 50-mL plastic tubes (Sarstedt, Germany). Twenty-five mL of KOH 10% were added to the roots before incubation at 70°C in a water bath for 45 min. The KOH was then removed, and roots were washed with HCl 1%. The staining step consisted in adding 25 mL of ink 2% (Parker blue ink, United States) in HCl 1%. The tubes were then placed at 70°C in a water bath for 30 min. The roots were rinsed and stored in deionized water before analysis. The total colonization rate was quantified using the gridline intersect method (Giovannetti & Mosse, 1980). Positive counts for AM colonization included the presence of vesicles or arbuscules or typical mycelium within the roots.

2.4 Gas Exchange Measurements

Gas exchange was monitored in fully expanded leaves at the plant vegetative stage. Net CO_2 fixation rate (Amax, μmol CO_2 m^{-2} s^{-1}), stomatal conductance to water vapor (gs, mmol H_2O m^{-2} s^{-1}), and transpiration rate (E, mmol H_2O m^{-2} s^{-1}) were measured in steady-state under conditions of saturating light (800 μmol m^{-2} s^{-1}) and 400 ppm CO_2 with a LI-6400 instrument (LI-COR, Lincoln, NE, USA). The intrinsic water use efficiency (WUEi) of leaf gas exchange was calculated from the gas exchange data as Amax/E.

2.5 Chlorophyll content

Chlorophylls were extracted from 1 g of frozen escarole leaves (−80°C) with 25 mL of acetone solvent. Samples were homogenized and centrifuged at 5,000g for 6 min at 4°C. Subsequently, the optical density of the supernatant was measured spectrophotometrically at wavelengths of 663 and 645 nm. The contents of chlorophyll a and b were calculated according to the Nagata and Yamashita equations (1992; see Materials and Methods Chapter III).

2.6 Maximum potential quantum efficiency of PSII

On the leaf used for gas exchange, the ratio between the variable fluorescence from a dark-adapted leaf (Fv) and the maximal fluorescence from a dark-adapted leaf (Fm), which is called the maximum potential quantum efficiency of PSII (Fv/Fm), was determined with a portable modulated fluorometer, model OS-30P (Opti-Science, Hudson, NY, USA). This ratio is the one of the most widely used in research employing the fluorescence technique and is considered a good indicator of the in vivo functionality of the photosynthetic apparatus in plants (Maxwell & Johnson, 2000). A special leaf clip holder was allocated to each leaf to maintain dark conditions for at least 30 min before reading.

2.7 Plant mineral content

Full-sized leaves and root fragments of each plant were freeze-dried for 72 h at −55°C (Christ Alpha 1-2 LDplus, Osterode am Harz, Germany). Anions were extracted with bidistilled water and were subsequently measured with an ion chromatograph (METROHM 861 Advanced Compact IC; METROHM 838 Advanced Sampler); the column used was a METROHM Metrosep CARB1 150/4.0 mm. Cations were extracted by acid digestion, using an ETHOSONE microwave digestion system (Milestone Inc., Shelton, CT, USA) and analyzed by inductively-coupled plasma optical emission (ICP-OES, Varian Vista MPX, Palo Alto, CA, USA). Nutrient use efficiency (NUE) was calculated as the ratio between total plant dry weight (g) and total nutrient content in the plant (g) according to Gerloff & Gabelman (1983) in Baligar et al. (Baligar et al., 2001), as follows:

$$\mathrm{NUE} = \frac{\text{Plant dry weight (g)}}{\text{Nutrient content in tissue (g)}}$$

2.8 Hormone extraction and analysis

Cytokinins (trans-zeatin, tZ, zeatin riboside, ZR, and isopentenyl adenine, iP), gibberellins (GA_1, GA_3, and GA_4), indole-3-acetic acid (IAA), abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA) and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) were analyzed according to Albacete et al. (2015) and Großkinsky et al. (2014) with some modifications. Briefly, a total of 100 mg of plant material was grounded in liquid nitrogen and dropped in 0.5 mL of cold (−20°C) extraction mixture of methanol/water (80/20, v/v). Then, 10 μL of an internal standard mix, composed of deuterated hormones ([2H5]tZ, [2H5]tZR, [2H6]iP, [2H2]GA_1, [2H2]GA_3, [2H2]GA_4, [2H5]IAA, [2H6]ABA, [2H4]SA, [2H6]JA, [2H4]ACC, Olchemim Ltd., Olomouc, Czech Republic) at a concentration of 1 μg mL^{-1} each, were added to the extraction homogenate. Solids were separated by centrifugation (20,000xg, 15 min, 4°C) and re-extracted for 30 min at 4°C in additional 0.5 mL of the same extraction solution. Pooled supernatants were passed through Sep-Pak Plus C18 cartridges (SepPak Plus, Waters, Milford, MA, USA) to remove interfering lipids and part of plant pigments and evaporated at 40°C under vacuum to near dryness. The residue was dissolved in 0.2 mL methanol/water (20/80, v/v) solution using an ultrasonic bath. The dissolved samples were filtered through 13 mm diameter Millex filters with 0.22 μm pore size nylon membrane (Millipore, Bedford, MA, USA). 10 μL of filtered extract were injected in a U-HPLC-MS system consisting of an Accela Series U-HPLC (ThermoFisher Scientific, Waltham, MA, USA) coupled to an Exactive mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA) using a heated electrospray ionization (HESI) interface. Mass spectra were obtained using the Xcalibur software version 2.2 (ThermoFisher Scientific, Waltham, MA, USA). For the quantification of the plant hormones, calibration curves were constructed for each analysed component (1, 10, 50, and 100 μg L^{-1}) and corrected for 10 μg L^{-1} deuterated internal standards. Recovery percentages ranged between 92 and 95%.

2.9 Statistical analyses

The data were tested first for homogeneity of variance and normality of distribution. The significance of the treatment effects was determined by analysis of variance (ANOVA). The significance ($P<0.05$) of the differences between mean values were tested by Tukey's honestly significant difference (HSD). Principal component analyses (PCA) were also performed. The Varimax rotation method was used for loading-PCA, while score-PCA was graphically plotted as a Bi-Plot score. All statistical analyses were done using the SPSS software (Version 25.0, SPSS Inc., Chicago, IL, USA).

3. Results

3.1 Growth-related determinations and mycorrhizal colonization

The AMF *R. irregularis* successfully colonized roots of escarole plants evaluated (by 60% on average, Figure 1a). No AMF colonization was detected in NM plants. The use of mulching did not produce any difference in the percentage of root colonization compared to plants without any cover.

Plant growth was significantly affected by the application of mulching and AMF. The use of mulching increased total FW, especially in AM plants, but this increment was only significant in escarole plants mulched with MS treatment (by 41%, Figure 1b). Similarly, shoot FW was significantly higher in mycorrhized plants grown under MS treatment in comparison with uncovered plants (by 32%, Figure 1c). Root FW and leaf area significantly increased (by 64% and 29%, respectively) in mycorrhized plants grown with MS treatment (Figure 1d,e). In contrast, although the use of AMF produced a slight increase in the number of leaves in mulched plants, it was not significant (Figure 1f).

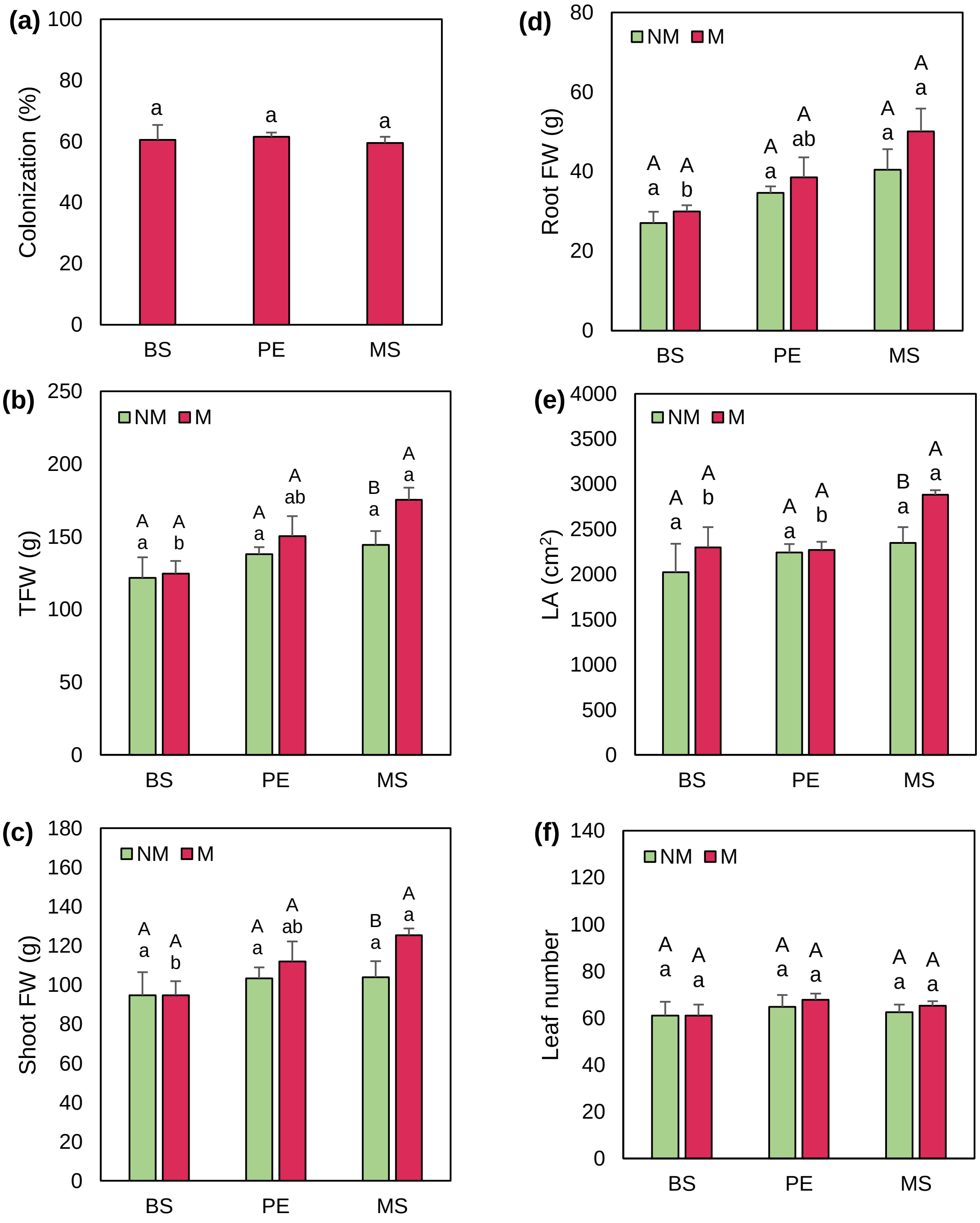

Figure 1. (a) Total fresh weight (FW), (b) shoot FW, (c) root FW, (d) leaf area (LA), (e) leaves number, and (f) percentage of colonization of escarole plants of the commercial variety "Bekele" cultivated under non-mycorrhizal (NM) and mycorrhizal (M) conditions and covered or not with mulching treatments. Bars show the means of five plants ± standard error. Different capital letters indicate significant differences due to the water stress treatment while different small letters show significant differences among mulching treatments according to Tukey's test ($P \leq 0.05$). Abbreviations used: non-covered treatment (BS), polyethylene mulch (PE) and mushroom substrate-based hydromulch (MS).

3.2 Gas Exchange Measurements

The combined use of AMF and mulching also produced differential leaf gas-exchange responses (Figure 2). In non-mycorrhizal plants, A_{CO2} increased over time both in mulched plants and non-mulched plants, but, in general, plants covered with PE presented higher levels throughout the whole period (Figure 2a). Interestingly, the A_{CO2} also increased over time by the use of AMF, with higher absolute values than those of NM plants, but this increase was only significant in plants mulched with PE at the last time-point. Interestingly, non-mycorrhizal plants presented the lowest values of photosynthesis during the studied period, which were significantly different in non-mulched plants 52 days after transplant. Stomatal conductance also increased over time, and this increase was especially apparent in AM plants grown under PE treatment at the last analytical time-point (Figure 2b). During the first 30 days of the experimental period, AMF did not produce any effect in terms of stomatal conductance, though the increase occurred from day 45 onwards. Importantly, although no differences were observed among mulching treatments, mycorrhized plants grown under PE and MS treatments showed higher stomatal conductance at the end of the experimental period (Figure 2b). The transpiration rate was strongly affected by the use of AMF over time (Figure 2c). Notably, this gas-exchange parameter reached the maximum value in mycorrhized plants grown under PE treatment, whereas non-mulched plants presented the lowest values of transpiration at the last time-point of the considered period (Figure 2C). Intrinsic water use efficiency (WUEi), calculated as the ratio between photosynthesis and transpiration rate, was higher at the beginning of the experiment and decreased during the considered period (Figure 2d). Importantly, mycorrhized plants presented in general the highest levels of WUEi, especially at the end of the studied period, but only non-covered plants presented significant differences compared to non-mycorrhized plants (Figure 2d).

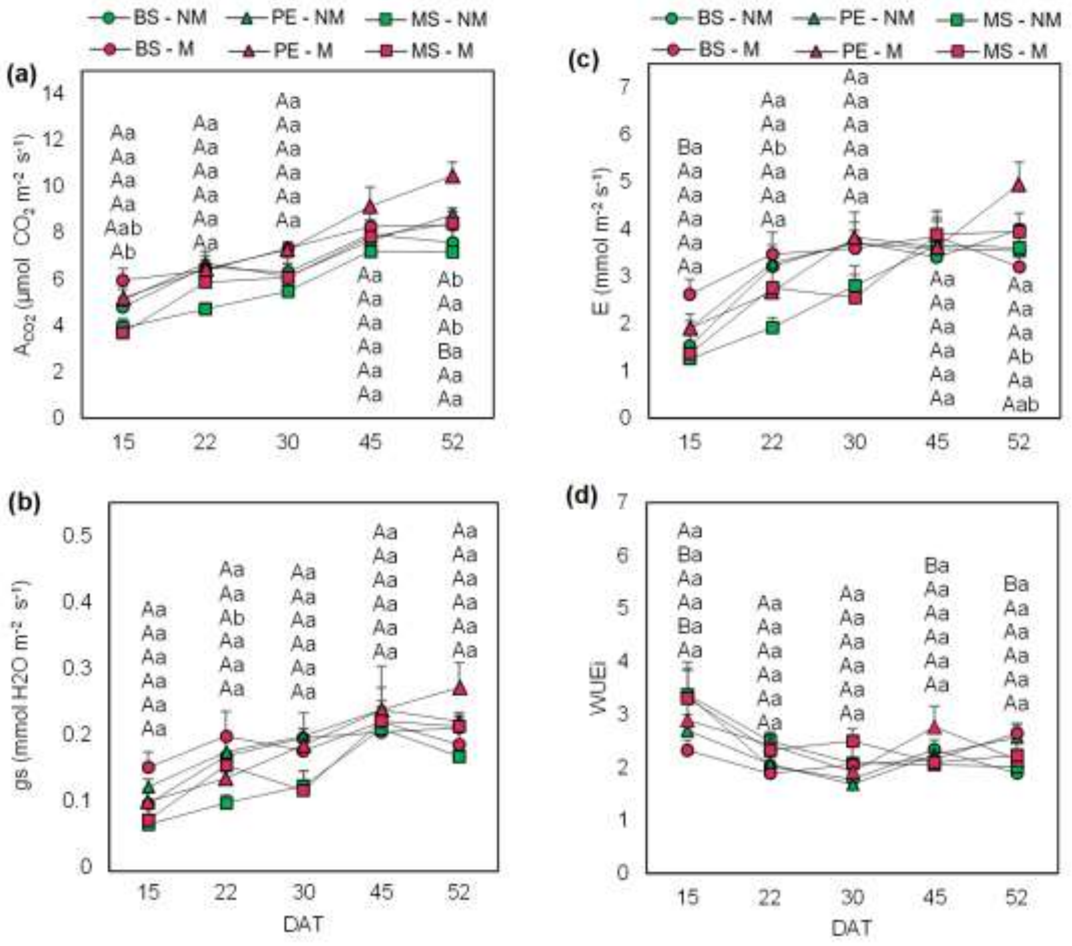

Figure 2. (a) Evolution of photosynthetic rate (A), (b) stomatal conductance (gs), (c) transpiration rate (E) and (d) intrinsic water use efficiency (WUEi) in escarole plants of the commercial variety "Bekele" cultivated under non-mycorrhizal (NM) and mycorrhizal (M) conditions and covered or not with mulching treatments. Bars show the means of five plants ± standard error. Different capital letters indicate significant differences due to the water stress treatment while different small letters show significant differences among mulching treatments according to Tukey's test ($P \leq 0.05$). Abbreviations used: non-covered treatment (BS), polyethylene mulch (PE) and mushroom substrate-based hydromulch (MS), days after transplanting (DAT).

3.3 Chlorophyll content and fluorescence

Mycorrhized plants mulched with PE presented a slightly higher content of chlorophyll a in comparison with the other treatments (by 15% on average), although this increase was not significant (Figure 3a). The same trend was observed in chlorophyll b concentrations (Figure 3b). However, even though no differences were observed in chlorophyll b, an increase was subtly observed in mycorrhized plants (by 8% on average) compared to non-mycorrhized plants. Regarding, chlorophyll fluorescence (Fv/Fm), the use of mulching and AMF inoculation did not produce any effect on this photosynthetic parameter (Figure 3c).

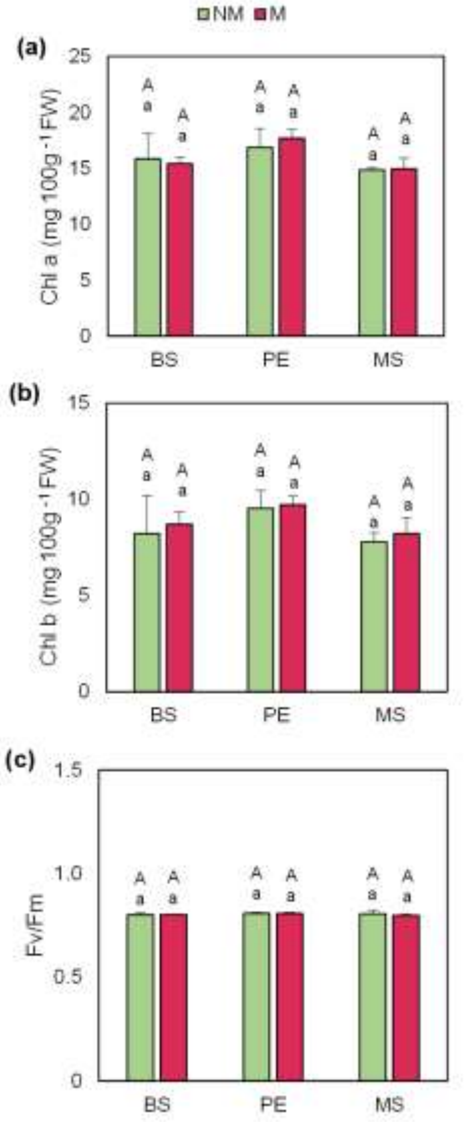

Figure 3. (a) chlorophyll a, (b) chlorophyll b, and (c) Chlorophyll fluorescence (Fv/Fm) in the leaves of escarole plants of the commercial variety “Bekele” cultivated under non-mycorrhizal (NM) and mycorrhizal (M) conditions and covered or not with mulching treatments. Bars show the means of five plants ± standard error. Different capital letters indicate significant differences due to the water stress treatment while different small letters show significant differences among mulching treatments according to Tukey’s test ($P \leq 0.05$). Abbreviations used: non-covered treatment (BS), polyethylene mulch (PE) and mushroom substrate-based hydromulch (MS).

3.4 Plant mineral content and nutrient use efficiency

The two experimental factors of this study, mulching and AMF inoculation, produced a differential ionic profile in the leaves and roots of escarole plants. Regarding leaves, P^{5+} concentrations were significantly lower in the mycorrhized plants (over 40% on average, Table 1). This can be explained by the use of Hoagland-P^{low} solution. Interestingly, AMF inoculation increased SO42- and reduced Na^+ concentrations, but these changes were only significant in PE-mulched plants (Table 1). Furthermore, the use of AMF or mulching treatment alone as well as their interaction decreased NO^{3-}, Mg^{2+}, and Cu^{2+} concentrations in leaves of escarole plants (Table 1). Likewise, the inoculation with AMF was found to decrease Na^+ and Ca^{2+} compared to non-mycorrhized plants. Importantly, AMF inoculation provoked a general ionic accumulation in the roots of escarole plants (Table 2). In general, mycorrhized plants showed an increase in K^+, Mg^{2+}, Ca^{2+}, Cu^{2+}, Mn^{2+}, and B^{3+} concentrations, but this increase was only significant in plants mulched with MS (Table 2). These results suggest that there exists an interaction between the MS hydromulching and the inoculation with AMF.

Table 3 shows the nutrient use efficiency of escarole plants. Interestingly, there existed a clear interaction between AMF inoculum and the mulching treatment for nitrogen use efficiency (NUE) and phosphorous use efficiency (PUE). Plants mulched with MS presented a significant increase in NUE both in non-mycorrhyzal and mycorrhizal plants in comparison with uncovered plants (by 3.2-fold and 5.6-fold, respectively). Furthermore, the same trend occurred in PUE, but only non-mycorrhizal plants presented significant differences among the mulching treatments (Table 3). Potassium use efficiency (KUE) was not affected by either the mulching treatment or the inoculation with AMF.

Table 1. Mineral nutrient concentrations of escarole leaves (cv. Bekele) cultivated under non-mycorrhizal (NM) and mycorrhizal (M) conditions and covered or not with mulching treatments.

AMF	Mulch	NO_3^- (mg g^{-1} DW)	P^{5+} (mg g^{-1} DW)	K^+ (mg g^{-1} DW)	Mg^{2+} (mg g^{-1} DW)	Ca^{2+} (mg g^{-1} DW)	SO_4^{2-} (mg g^{-1} DW)
NM	BS	22.83±4.18 Aa	5.65±0.44 Aa	51.51±1.91 Ab	6.06±0.64 Aa	6.44±0.85 Aa	7.81±1.83 Aa
	PE	14.22±3.15 Aa	5.57±0.40Aa	54.50±1.22 Aab	5.35±0.80 Aa	7.35±0.54 Aa	7.56±1.03 Ba
	MS	12.84±2.10 Aa	5.07±0.29 Aa	58.44±1.87 Aa	3.80±0.14 Aa	8.33±0.22 Aa	7.64±0.95 Aa
M	BS	7.60±0.45 Ba	3.14±0.32 Ba	54.20±2.68 Aa	5.89±0.62 Ba	6.72±0.93 Aa	9.52±1.06 Aa
	PE	7.19±0.92 Aa	2.84±0.34 Ba	54.54±2.33 Aa	4.96±0.34 Aab	6.86±0.33 Aa	12.67±1.67 Aa
	MS	2.06±0.05 Bb	2.71±0.27 Ba	53.99±2.90 Aa	3.84±0.29 Ab	9.18±0.81 Aa	7.61±1.32 Aa

AMF	Mulch	Cu^{2+} (mg kg^{-1} DW)	Mn^{2+} (mg kg^{-1} DW)	Zn^{2+} (mg kg^{-1} DW)	B^{3+} (mg kg^{-1} DW)	Na^+ (mg g^{-1} DW)
NM	BS	6.43±0.96 Aa	44.75±50.92 Aa	95.34±10.03 Aa	37.55±3.03 Aa	6.33±0.97 Aa
	PE	5.47±0.38 Aa	34.99±32.19 Aa	90.74±9.91 Aa	38.42±4.79 Ba	6.67±1.11 Aa
	MS	4.65±0.22 Aa	42.06±47.53 Aa	78.72±5.49 Aa	34.93±5.49 Aa	5.23±0.34 Aa
M	BS	7.20±0.72 Ba	41.12±27.92 Aa	100.49±10.44 Aa	27.48±4.37 Aa	4.81±S0.83 Aa
	PE	5.90±0.29 Aab	32.85±25.06 Aa	87.25±13.54 Aa	32.02±4.44 Aa	3.46±0.35 Ba
	MS	4.25±0.78 Ab	31.49±35.15 Aa	64.15±9.41 Aa	36.74±3.64 Aa	5.12±0.92 Aa

Different small letters within a row indicate significant differences between the mulching treatments, and different capital letters within a column, for the same mulching treatment, indicate significant differences between the use of mycorrhiza (P = 0.05, HSD Tukey test). Abbreviations used: boron (B^{3+}), calcium (Ca^{2+}), Copper (Cu^{2+}), potassium (K^+), magnesium (Mg^{2+}), manganese (Mn^{2+}), nitrogen (N), sodium (Na^+), phosphorous (P^{5+}), sulphate (SO_4^{2-}), zinc (Zn), non-covered treatment (BS), polyethylene mulch (PE) and mushroom substrate-based hydromulch (MS).

Table 2. Mineral nutrient concentrations of escarole root (cv. Bekele) cultivated under non-mycorrhizal (NM) and mycorrhizal (M) conditions and covered or not with mulching treatments.

AMF	Mulch	NO_3^- (mg g^{-1} DW)	P^{5+} (mg g^{-1} DW)	K^+ (mg g^{-1} DW)	Mg^{2+} (mg g^{-1} DW)	Ca^{2+} (mg g^{-1} DW)	SO_4^{2-} (mg g^{-1} DW)
NM	BS	5.53±0.56 Aa	7.26±0.91 Aa	41.49±5.10 Aa	4.76±0.73 Aa	6.19± 0.71Aa	14.91±1.34 Ab
	PE	5.97±0.77 Aa	8.44±0.32 Aa	51.27±1.12 Aa	5.28±0.24 Aa	6.66±0.32 Aa	12.32±0.69 Ab
	MS	4.08±1.08 Aa	3.92±0.38 Ab	39.58±3.49 Ba	4.16±0.48 Ba	5.99±0.68 Ba	22.24±0.71 Aa
M	BS	2.61±0.08 Aa	1.93±0.49 Ba	70.39±1.52 Aa	7.45±1.29 Aa	8.54±1.63 Aa	18.64±0.80 Bab
	PE	2.66±0.23 Ba	1.45±0.10 Ba	50.76±3.87 Aa	5.52±0.22 Aa	7.40±0.44 Aa	14.11±1.25 Bb
	MS	1.22±0.17 Ab	2.08±0.36 Ba	87.47±1.49 Aa	8.36±1.10Aa	10.33±1.37 Aa	24.18±2.56 Aa

AMF	Mulch	Cu^{2+} (mg kg^{-1} DW)	Mn^{2+} (mg kg^{-1} DW)	Zn^{2+} (mg kg^{-1} DW)	B^{3+} (mg kg^{-1} DW)	Na^+ (mg g^{-1} DW)
NM	BS	20.56±1.03 Aab	81.23±5.94 Aa	30.03±2.89Aa	13.76±2.26 Aa	8.29±0.81 Aab
	PE	22.72±8.83 Aa	68.12±4.95 Aab	38.22±0.70 Aa	18.10±0.66 Aa	9.12±0.33 Aa
	MS	12.65±0.85 Bb	59.77±2.93 Aa	29.37±3.24 Aa	12.35±1.31 Ba	6.53±0.62 Bb
M	BS	27.37±6.25 Aa	114.85±8.25 Aa	34.85±2.67 Aa	21.65±2.77 Aa	11.19±2.51 Aa
	PE	35.42±7.85 Aa	63.75±5.20 Aa	39.15±3.77 Aa	20.57±0.57 Aa	10.22±0.72 Aa
	MS	22.75±2.77 Aa	187.35±10.11 Ba	45.85±1.35 Ab	25.47±1.81 Aa	11.07±1.68 Aa

Different small letters within a row indicate significant differences between the mulching treatments, and different capital letters within a column, for the same mulching treatment, indicate significant differences between the use of mycorrhiza (P = 0.05, HSD Tukey test). Abbreviations used: boron (B^{3+}), calcium (Ca^{2+}), Copper (Cu^{2+}), potassium (K^+), magnesium (Mg^{2+}), manganese (Mn^{2+}), nitrogen (N), sodium (Na^+), phosphorous (P^{5+}), sulphate (SO_4^{2-}), zinc (Zn), non-covered treatment (BS), polyethylene mulch (PE) and mushroom substrate-based hydromulch (MS).

Table 3. Nutrient use efficiency of escarole plants (cv. Bekele) cultivated under non-mycorrhizal (NM) and mycorrhizal (M) conditions and covered or not with mulching treatments.

AMF	Mulch	NUE	PUE	KUE
NM	BS	25.03±2.58 Bb	802.94±93.98 Bb	108.74±6.90 Aa
	PE	39.02±6.32 Bab	717.66±29.99 Bb	94.66±1.88 Aa
	MS	59.28±7.97 Ba	1127.48±75.91 Ba	102.22±2.56 Aa
M	BS	90.88±3.73 Ab	1985.08±82.96 Aa	83.02±8.21 Aa
	PE	91.08±15.97 Ab	2346.22±105.89 Aa	95.19±2.74 Aa
	MS	335.93±26.96 Aa	1290.88±280.68 Aa	74.01±9.04 Ba

Different small letters within a row indicate significant differences between the mulching treatments, and different capital letters within a column, for the same mulching treatment, indicate significant differences between the use of mycorrhiza (P = 0.05, HSD Tukey test). Abbreviations used: nitrate use efficiency (NUE), phosphorous use efficiency (PUE), potassium use efficiency (KUE), non-covered treatment (BS), polyethylene mulch (PE) and mushroom substrate-based hydromulch (MS).

3.5 Hormonal profile

Figure 4 shows the GA profile in escarole plants, composed of GA_3 and GA_4. The balance of the two most active GAs were affected by the use of AMF and the mulching treatment (Figure 4). GA_3 was the most abundant GA in escarole leaves. MS treatment provoked a significant increase in the endogenous GA_3, but, in this case, it was especially apparent in the AMF inoculated plants in comparison with inoculated plants without cover (5.7-fold, Figure 4a). GA_4 followed the same trend, since the use of MS mulch provoked a strong increase of this hormone in mycorrhyzed plants in comparison to non-covered plants (7.7-fold, Figure 4b). Consequently, total GA concentrations, calculated as the sum of GA_3 and GA_4, were significantly higher in mycorrhized plants mulched with MS, while the leaves of non-mulched plants presented the lowest total GA concentrations (Figure 4c).

Three of the most active CKs in higher plants, tZ, RZ, and iP, were analyzed in escarole leaves, but only tZ and iP concentrations were detected (Figure 5). The concentrations of tZ were strongly affected by the use of mulching, both in mycorrhized and non-mycorrhized plants (Figure 5a). The use of AMF provoked an increase both in PE and MS mulched plants (2.0 and 2.2-fold respectively) in comparison with those without cover. For iP, the highest concentration was also found in the interaction between AMF and MS mulch (by 72%, Figure 5b). Total CK concentrations, calculated as the sum of tZ and iP, was significantly higher in mycorrhized plants mulched with MS treatment (2.3-fold higher than non-covered plants), followed by plants covered with plastic (Figure 5c). Regarding auxins, the most active one in higher plants, IAA, was detected in all treatments. Even though no significant differences were observed between the different mulching treatments, plants mulched with MS presented a significant increase in IAA in presence of AMF inoculum (Figure 6a).

ABA has been linked traditionally with a wide range of plant stress responses. ABA concentrations were heterogeneous depending on the mulching treatment and the presence of AMF inoculum (Figure 6b). In non-mycorrhized plants, the lowest concentrations of this hormone were found in PE-mulched plants (by 30% lower on average). When plants were grown with AMF inoculum, plants without cover significantly increased ABA concentrations compared to plants mulched with PE (Figure 6b). The important role of ethylene as a stress-related hormone is widely recognized and its direct precursor, ACC, has been measured in leaves of escarole (Figure 6c). The use of AMF decreased ACC concentrations in all mulching treatments, but plants mulched with MS presented significantly lower ACC concentrations (by 30%) than non-mulched plants with or without AMF inoculum. SA concentrations were strongly affected by the use of mulching (Figure 6d) both in mycorrhized and non-mycorrhized plants. Even though no significant differences were observed, plants without cover showed a decrease in SA concentrations both in the presence and absence of AM inoculum in comparison with those mulched with PE and MS (Figure 6d). AMF inoculation also provoked a general increase in JA concentrations, which was more evident in BS and MS plants (Figure 6E). Similarly to SA, the use of mulching affected JA concentrations, especially in mycorrhized plants grown under MS treatment (2.3-fold higher) compared to those without cover (Figure 6e).

To summarize the changes in the hormonal balance of escarole leaves provoked by the combination of different mulching treatments and the inoculation with AMF, a cluster heat plot was performed (Figure S1). As aforementioned, the hormonal profile studied was clearly affected both by the type of mulch and by the presence or absence of AMF inoculum. The results indicate that GAs and CKs were the hormones that augmented in mycorrhizal plants subjected to mulching treatments, especially in MS plants. In contrast, ACC and ABA decreased in mulched plants, particularly in those inoculated with AMF (Figure S1).

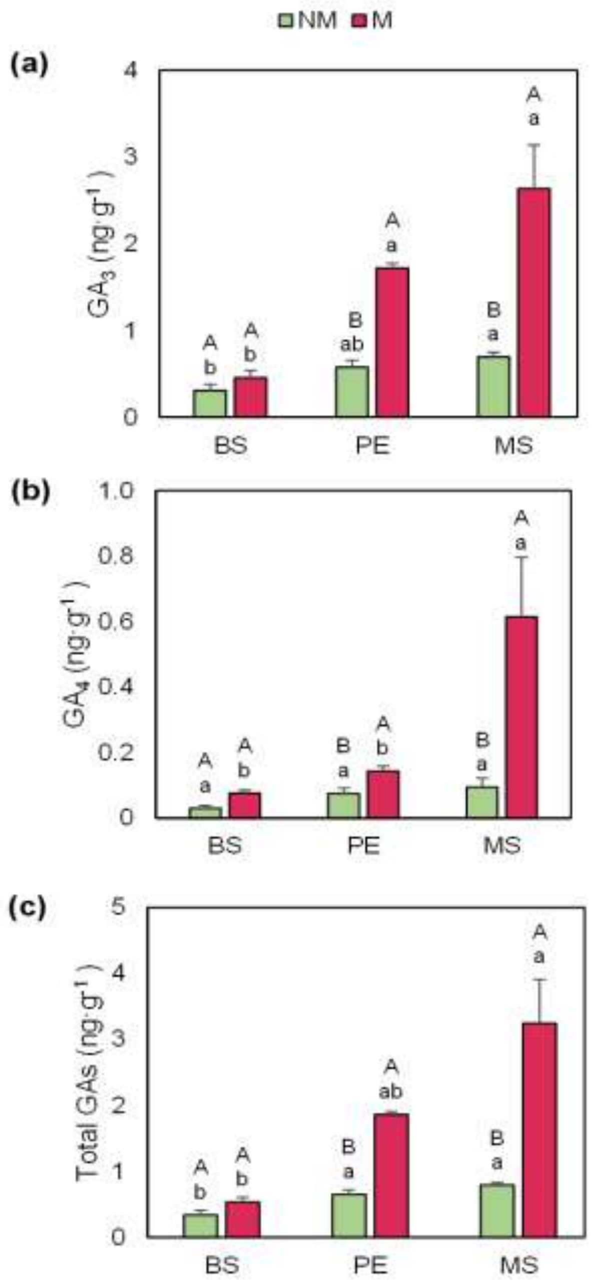

Figure 4. (a) gibberellin A3 (GA_3), (b) gibberellin A4 (GA_4) and (c) total gibberellin (GAs) concentrations in leaves of escarole plants of the commercial variety "Bekele" cultivated under non-mycorrhized (NM) and mycorrhized (M) conditions and covered or not with mulching treatments. Bars show the means of five plants ± standard error. Different capital letters indicate significant differences due to the water stress treatment while different small letters show significant differences among mulching treatments according to Tukey's test ($P \leq 0.05$). Abbreviations used: bare soil (BS), polyethylene mulch (PE) and mushroom substrate-based hydromulch (MS).

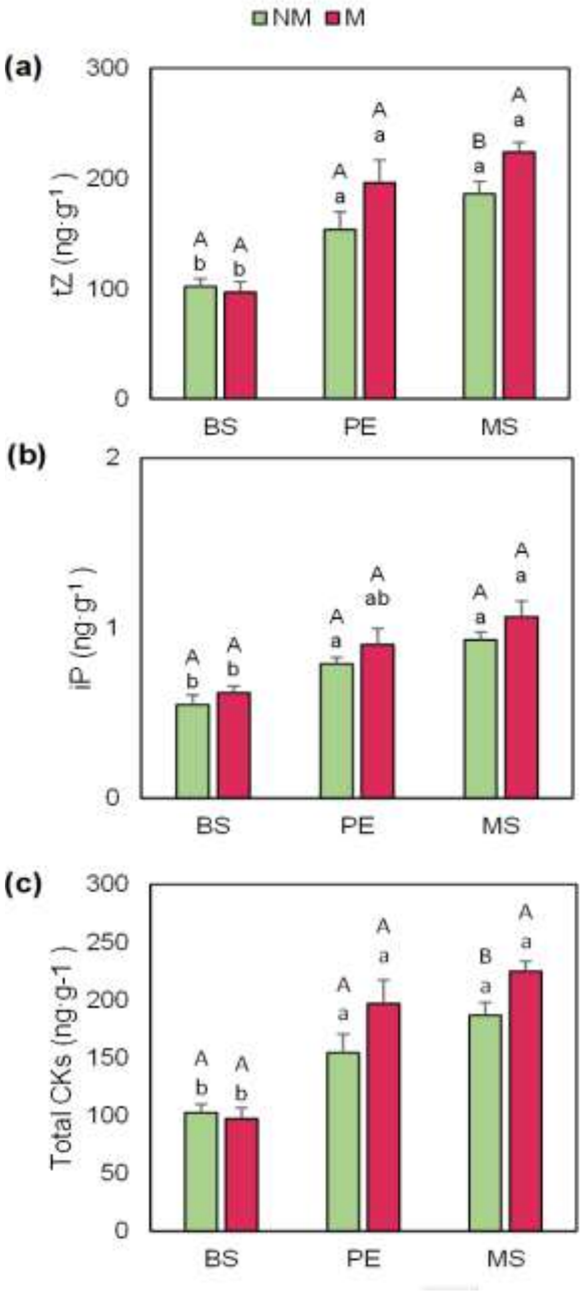

Figure 5. (a) Trans-zeatin (tZ), (b) isopentenyladenine (iP) and (c) total cytokinin (CKs) concentrations in leaves of escarole plants of the commercial variety "Bekele" cultivated under non-mycorrhized (NM) and mycorrhized (M) conditions and covered or not with mulching treatments. Bars show the means of five plants ± standard error. Different capital letters indicate significant differences due to the water stress treatment while different small letters show significant differences among mulching treatments according to Tukey's test ($P \leq 0.05$). Abbreviations used: bare soil (BS), polyethylene mulch (PE) and mushroom substrate-based hydromulch (MS).

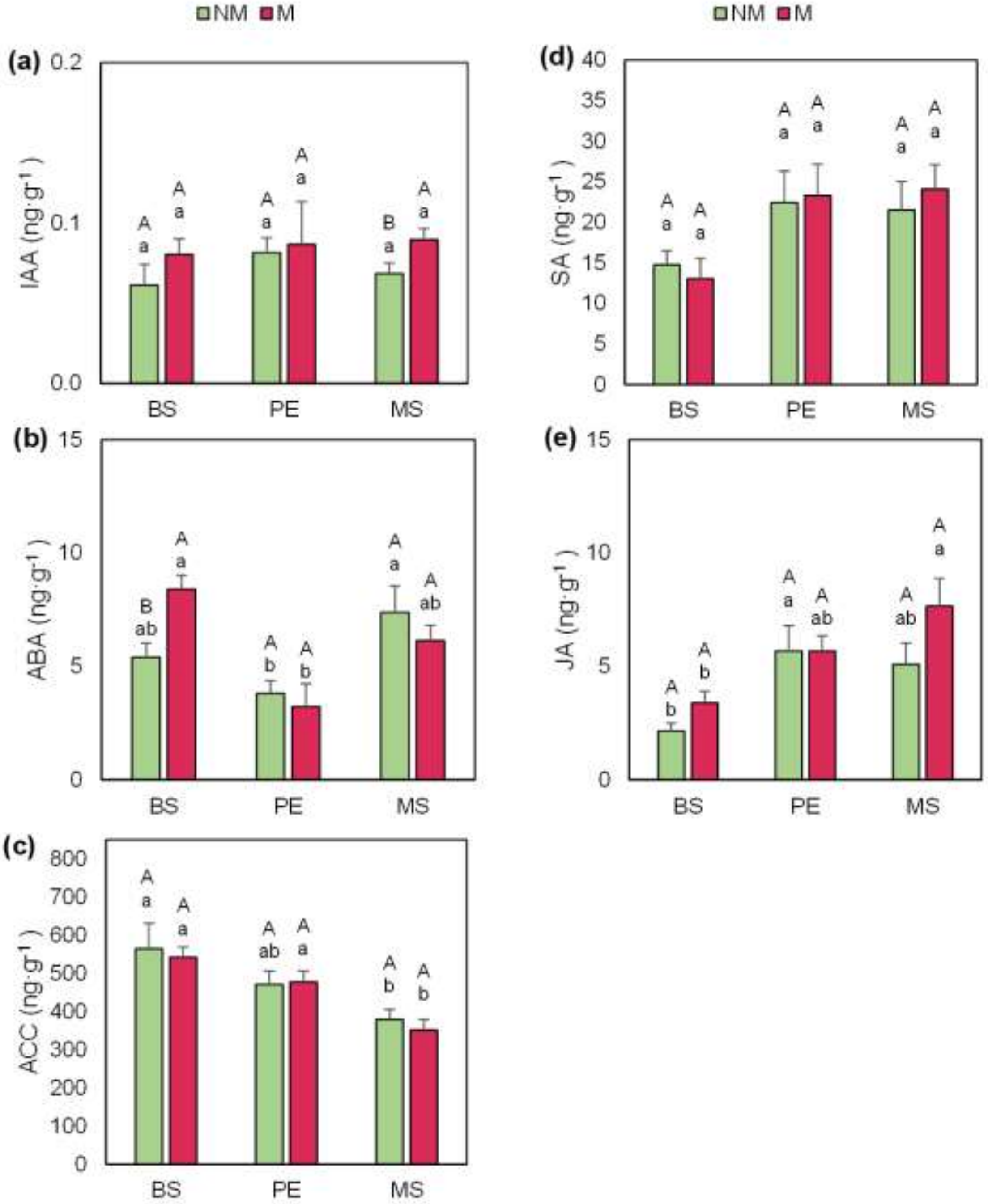

Figure 6. (a) 1-aminocyclopropane-1-carboxylicacid (ACC), (b) abscisic acid (ABA), (c) Indole acetic acid (IAA), (d) salicylic acid (SA) and (e) jasmonic acid (JA) concentrations in leaves of escarole plants of the commercial variety "Bekele" cultivated under non-mycorrhized (NM) and mycorrhized (M) conditions and covered or not with mulching treatments. Bars show the means of five plants ± standard error. Different capital letters indicate significant differences due to the water stress treatment while different small letters show significant differences among mulching treatments according to Tukey's test ($P \leq 0.05$). Abbreviations used: bare soil (BS), polyethylene mulch (PE) and mushroom substrate-based hydromulch (MS).

3.6 Principal component analysis

In order to identify important parameters associated with the variability factors studied, the data set was subjected to a score PCA (Figure 7a). This statistical test transforms the normalized data into principal component scores through multiple dimension rotation (Albacete et al., 2010). The score PCA showed a clear separation between the scores of the escarole plants evaluated with and without AMF inoculum and among the different mulching treatments. The scores of plants grown without AMF inoculum were clearly separated from those with AMF inoculum (Figure 7a). Importantly, MS treatment provoked a strong separation of the scores of mycorrhized plants compared to the other clusters.

Furthermore, a loading PCA was performed to reduce the dimensionality of the data set with the loadings of the variables used in this study (Figure 7b). This mathematical algorithm allows identifying important ionic and hormonal traits regarding the growth and productivity-associated characteristics of escarole plants by the inoculation with AMFand the mulching treatment while maintaining the statistical variability (Albacete et al., 2010). The explained variability, which is the sum of that from the principal component PC1 and PC2, was around 50%. Importantly, the loading PCA revealed that the growth parameters (shoot FW, root FW, leaf number, and leaf area) were closely associated with each other and with important ionic (root SO_4^{2-} and leaf Ca^{2+}) and hormonal (JA, total GAs and total CKs) factors (Figure 7b). Furthermore, NUE and PUE were coupled with all growth variables studied. In contrast, the ionic (leaf and root NO^{3-}, leaf and root P^{5+} and leaf Cu^{2+}, Zn^{2+} and Mg^{2+}) and the ethylene precursor ACC were inversely associated with the productivity traits of escarole plants (Figure 7B).

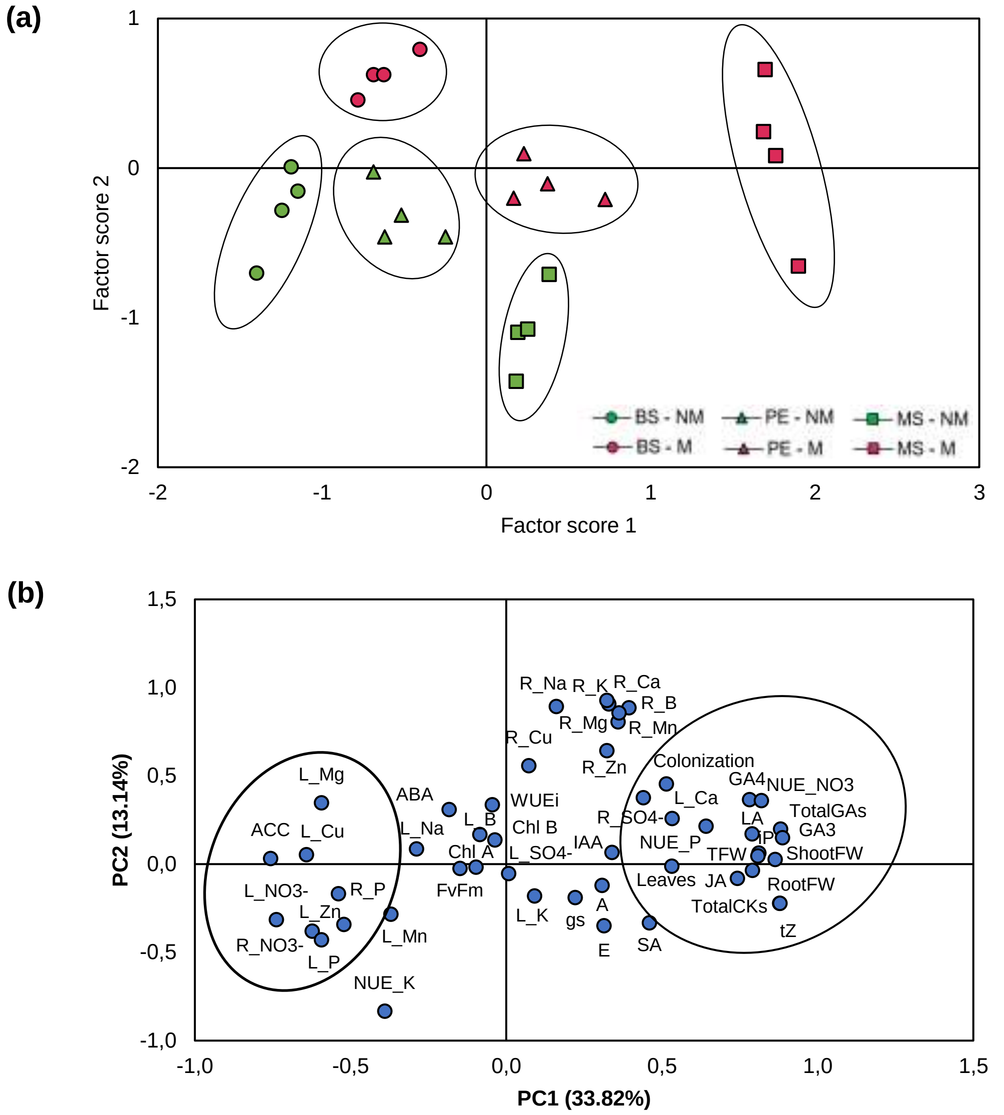

Figure 7. (a) Bi-Plot representing the score values and (b) two axes of a principal component (PC1, PC2) analysis showing the loadings of various growth-related, ionic and hormonal variables (denoted by abbreviations) of the escarole commercial variety "Bekele" non-mulched or subjected to different mulching treatments and cultivated under non-mycorrhized (NM) and mycorrhized (M) conditions. Circles enclose those variables/scores which cluster together in loading PCA and score PCA. Abbreviations used: boron (B), calcium (Ca), copper (Cu), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), phosphorus (P), sulfate (SO4^{2-}), nitrate (NO$_3^-$), nitrate nutrient use efficiency (NUE-NO$_3^-$), phosphorous nutrient use efficiency (NUE-P), potassium nutrient use efficiency (NUE-K), root fresh weigh (RootFW), shoot fresh weight (ShootFW), leaf area (LA), total fresh weight (TFW), chlorophyll a (Chl a), chlorophyll b (Chl b), net CO$_2$ fixation rate (A), stomatal conductance (gs), transpiration rate (E), intrinsic water use efficience (WUEi), abscisic acid (ABA), 1-aminocyclopropane-1-carboxylic acid (ACC), indole acetic acid (IAA), salicylic acid (SA), jasmonic acid (JA), gibberellin A3(GA$_3$), gibberellin A4 (GA$_4$), total gibberellins (GAs), trans-zeatin (tZ), isopentenyladenine (iP), total cytokinins (CKs).

4. Discussion

With the rapid increase of the world's population, there is a corresponding rise in food demand which is followed by concerns about the stability of the global environment (Pareek et al., 2020). Some strategies such as the use of AMF or the mulching technology have been used to improve plant growth and performance and crop productivity (Begum et al., 2019; Kader et al., 2017; Raklami et al., 2019; Song et al., 2020). The combined use of AMF and ecological mulching could be a sustainable strategy to increase plant production in horticultural crops, especially in the Mediterranean basin, which is one of the most important horticultural areas in Europe. Previously, we have described growth developmental changes of mulched escarole plants grown under water limitations (Romero-Muñoz et al., 2022). We found that ecological hydromulching improved growth and productivity under water stress, and this was related to the capacity of the plant to regulate water relations and CO_2 assimilation through fine-tuning stomata opening due to the antagonistic interaction of CKs and ABA (Romero-Muñoz et al., 2022). In the present study, escarole plants were assayed under the presence or absence of the AMF *Rhizophagus irregularis* and with different mulching treatments. We have found that the inoculation with AMF increased the growth of escarole plants in all mulching treatments. Importantly, the MS treatment had an additional positive effect, especially in shoot and root FW and leaf area (Figure 1c-e). Several studies confirm that mycorrhized roots can explore more soil volume due to their extramatrical hyphae, which facilitate the absorption and translocation of several nutrients (Guo et al., 2010; Joner & Jakobsen, 1995). Indeed, increments in phosphorous content in plant tissues have been reported due to enhanced uptake by the hyphae (Bonfante & Genre, 2010; Garcés-Ruiz et al., 2017). In our study, phosphorous concentrations in both shoot and root associated in an opposite cluster to that of the growth parameters within the PCA (Figure 7). Despite the lower phosphorous concentrations in mycorrhized plants by the use of Hoagland-P^{low} (Tables 1,2), *R. irregularis* improved phosphorous uptake in escarole plants, thus maintaining and/or increasing plant growth (Figure 1). It has been documented that under nutrient limitations, plants increase their radicular structure to facilitate ionic absorption and avoid nutrient imbalance (Zhang et al., 2021), while other authors also reported that root system morphological development can be affected by soil nutrient status, and also by AMF (Wu et al., 2016). Furthermore, the use of organic mulches is also related to a higher area of the root zone area (Fausett & Rom, 2001). This could explain the increase in most macro and micronutrients in the roots of AMF plants, especially in those hydromulched with MS (Table 2). Importantly, the growth improvement provoked by AMF inoculation (Table 3) was associated with phosphorous and nitrogen use efficiencies in the loading-PCA (Figure 7b). The study of Zhu et al. (2016) informed that wheat plants grown at elevated CO_2

presented significant increases of nitrogen use efficiency compared when these plants were inoculated with AMF. Furthermore, Liu et al. (2019) demonstrated that AMF symbiosis had a positive effect in nitrogen use efficiency of soybean grown under water stress.

In addition to providing nutritional and structural benefits to plants, AMF also awards other benefits including production/accumulation of secondary metabolites, increased resistance against biotic and abiotic stresses and enhanced photosynthesis rate (López-Ráez et al., 2010; Ruiz-Lozano, 2003; Sheng et al., 2008). According to Begum et al. (2019), increased photosynthetic activities are directly related to improved growth frequency of AMF inoculation. Indeed, in our study, the combination of AMF inoculation and mulch increased the gas exchange parameters of escarole plants, especially at the end of the experimental period (Figure 2). As stated above the highest gas exchange related parameters in PE mulched plants may be due to the physical properties of this material. The impermeability of this material blocked substrate evaporation, and thus these plants had to increase stomatal opening to facilitate plant water (and gas) exchange, resulting in a rise of the photosynthetic activity. Recently, Song et al. (2020) have shown in wheat mulched plants that the use of plastic films strongly increases the net photosynthetic rate. Niu et al. (2020) also described the positive effect of plastic mulch on photosynthetic rate and dark respiration rate due mainly to the higher stomatal density and aperture area of maize leaves. The interaction of AMF inoculation and mulching could be explained by a direct effect on the hormonal balance of the plant. Indeed, we found the lowest endogenous ABA levels in plants mulched with PE treatment inoculated with AMF (Figure 6b), which presented the highest stomatal conductance (Figure 2b). In lettuce plants colonized by *R. intraradices,* it has been shown lower ABA levels than non-AMF plants, thus maintaining stomatal opening under salinity conditions (Jahromi et al., 2008). In contrast, the study of Khalloufi et al. (2017) informed about the increase of endogenous ABA concentration in tomato plants inoculated with *R. irregularis* both under control and salinity conditions. Taking this into account, it might be suggested that the effect of AMF on ABA content could vary depending on the host plant and/or the experimental conditions.

Importantly, in our study, the growth-related hormones GAs and CKs were also affected, thus enhancing plant development (Figures S1 and 7b). A specific mulching response was found regarding GA leaf content (Figures 4 and S1), especially in MS mulching treatment. In this regard, the growth-related parameters clustered with GA_3, GA_4 and total GAs in the loading-PCA (Figure 7B). Recently, the study of Romero-Muñoz et al. (2022) has stated that the levels of bioactive GAs increased in escarole plants mulched with MS. In addition, early reports in tobacco plants have revealed that AMF is also related in the production of some types of GAs, such as GA_1 and its deactivation product GA_8 (Shaul-Keinan et al., 2002). Likewise, the study of Khalloufi et al. (2017) found that the levels of GA_1 and GA_3 increased in leaves of mycorrhized tomato plants both under control and salinity conditions. Therefore, the interaction between GAs and AMF seems to be especially important to increase plant growth. CKs are classically closely related with plant growth responses (Wilkinson et al., 2012), and were also especially affected by the interaction of AMF inoculation and mulching. Indeed, the concentration of one of the most active CKs in plants, tZ, significantly increased in mycorrhized plants (Figures 5a and S1), especially in the AMF plants mulched with MS. Interestingly, these plants showed the highest shoot, root, and total FW alone or in combination with AMF inoculation (Figure 1b-d). Importantly, CKs also clustered with the growth-related parameters in the loading-PCA (Figure 7B). Early reports observed a strong correlation between enhanced growth, improved photosynthesis, and the increase of CKs levels in AMF plants (Drüge & Schonbeck, 1993; Shaul-Keinan et al., 2002). The study of Cosme et al. (2016) suggested that both shoot- and root-specific alterations of endogenous CK levels had key roles in the relation between CK homeostasis and growth in tobacco plants inoculated with AMF. Similarly, Miransari et al. (2014) reported that increased phosphate uptake in mycorrhized plants could be explained by the augmentation of root CK content and CK flux to the shoots, thus enhancing plant growth.

Several authors have addressed the possible role of auxins in plant growth (Barker & Tagu, 2000; Khalloufi et al., 2017). However, in the present study, we found that endogenous levels of IAA in escarole leaves were not affected by either AMF inoculation or the mulching treatment. Importantly, the endogenous levels of SA and JA increased with the use of mulching, but, notably, after AMF inoculation, especially in the MS treatment (Figure 6d,e). We found that JA clustered with the growth parameters (Figure 7B), but the correlation between SA and the growth-related parameters was unclear. This suggests a role for JA in the control of escarole growth. In this regard, some previous reports have shown the positive effect of JA on growth in AMF plants (López-Ráez et al., 2010; Miransari et al., 2014). Nevertheless, both SA and JA are related with biotic stress responses, indicating that the fungal hyphae colonization may provoke plant defense responses, which are compensated by the additional benefits on plant growth (Pozo et al., 2015).

5. Conclusions

In this study, we have tested the growth effects of a new mulching formulation called hydromulching in combination with the inoculation of the arbuscular mycorrhizal fungus *Rhizophagus irregularis* in a commercial escarole variety grown under controlled conditions. The mulching application and the AMF inoculation separately, but especially their interaction, enhanced the growth of escarole plants. The growth improvement observed has been explained by the regulation of the hormonal balance of the plant. In this regard, inoculated plants mulched with MS increased the most active GAs, especially GA_3, which clustered with all growth-related parameters studied. Likewise, the active CKs, tZ and iP, and JA concentrations increased with AMF inoculation or hydromulching application, but particularly by their interaction, which was associated with plant growth promotion. This work is hence an important step toward the use of sustainable strategies to improve horticultural crop production. This is especially relevant in the actual climate change context, since there exists an urgent need for sustaining food security, particularly in the Mediterranean basin, one of the most important horticultural areas in Europe.

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Conduding remarks

I. Hydromulching improved soil physicochemical and biological parameters, which induced a better water status of artichoke plants. This has a direct implication on the improvement of the artichoke crop productivity and physical quality characteristics of the heads.

II. The hydromulching treatments were more sustainable than plastic films in terms of soil fertility and nutrient cycling, provoking a general increase in artichoke head mineral content, while sugars decreased with respect to bare soil. This can be attributed to lower water retention of bare soil thus generating transient water stress that induced adaptative responses, such as sugar accumulation.

III. The effect of hydromulching on the growth and productivity responses of artichoke plants subjected to water deficit has been associated with improved photosynthesis and water use efficiency, and controlled by hormonal regulation of source-sink relationships. In this regard, the crosstalk of the growth-promoting hormones, CKs, with the stress-related hormones, ACC, ABA, and SA, has been shown to regulate sucrolytic activities in both source leaves and artichoke heads under drought stress

IV. The growth of hydromulched escarole plants was significantly improved under drought stress, explained by a specific effect on plant water relations and photosynthesis-related parameters regulated by hormones. Indeed, ABA content increased with drought in hydromulched plants but to a lower extent than in non-covered plants, thus controlling stomata opening, which, in crosstalk with ACC and SA, improved plant water relations through fine regulation of CKs/ABA balance.

V. The mulching application and the AMF inoculation separately, but especially their interaction, enhanced the growth of escarole plants explained by the regulation of the hormonal balance of the plant. The concentrations of CKs, GAs, and JA increased with AMF inoculation or hydromulching application, but particularly by their interaction, which was associated with plant growth promotion.

Appendix

Supplementary material

CHAPTER II

The use of hydromulching increases yield and quality of artichoke (*Cynara cardunculus* var. *scolymus*) by improving soil physicochemical and biological properties

Supplementary Table 1. Mean monthly air temperature and rainfall during the growing seasons of this study (2018 and 2019).

Growing months	Temperature 2018 (°C)	Temperature 2019 (°C)	Averaged temperature variation over the last 20 years (°C)	Rainfall 2018 (mm)	Rainfall 2019 (mm)	Averaged rainfall variation over the last 20 years (mm)
January	12.06	10.86	1.19	36.90	2.00	-13.23
February	10.73	11.82	0.26	14.00	0.10	-8.92
March	14.31	13.55	0.87	15.40	20.40	-5.80
April	16.5	15.61	0.69	7.10	116.40	35.14
May	19.07	19.15	0.59	8.20	1.50	-8.81
June	23.01	22.6	0.26	19.10	0.50	4.54
July	26.03	26.85	1.36	0.00	0.20	-1.05
August	27.18	26.54	1.37	0.10	15.90	-1.84
September	24.7	23.68	1.54	65.30	89.50	38.20
October	19.09	19.60	0.61	61.00	34.70	15.38
November	14.48	14.60	0.86	75.10	13.00	3.36
December	14.48	14.60	0.86	14.80	80.80	1.97

*Data collected from the climatic station at the IMIDA experimental field (latitude: 37° 46' N; longitude: 89" 0° 53' W) located in Murcia, Spain.

Supplementary Table 2. Chemical element concentrations in the soil and in the organic hydromulches at the beginning of the experimental period.

	N (mg g^{-1})	C (mg g^{-1})	P^{+5} (mg g^{-1})	K^{+} (mg g^{-1})	Mg^{2+} (mg g^{-1})	SO_4^{2-} (mg g^{-1})	Na^{+} (mg g^{-1})	Cl^{-} (mg g^{-1})
Soil	0.73 b	6.09 b	0.02 b	0.05 b	$4.14 \cdot 10^{-3}$ b	0.44 b	0.16 b	0.26 b
MS	15.13 a	296.76 ab	4.06 a	5.38 a	6.52 a	58.47 ab	1.36 a	2.35 ab
RH	2.49 ab	379.84 a	0.39 ab	1.18 ab	3.03 ab	68.36 a	0.70 ab	0.95 ab
WS	1.80 ab	365.16 a	0.28 ab	4.01 ab	4.13 ab	62.41 ab	1.32 a	3.39 a

All data are expressed as mean ± standard error, n = 4. Means were separated using Kruskal-Wallis test, P = 0.05. Different letters within a column indicate significantly (P = 0.05) differences between treatments. Abbreviations used: mushroom substrate-based hydromulch (MS), rice husk-based hydromulch (RH), wheat straw -based hydromulch (WS).

CHAPTER III

Hydromulching enhances the growth of artichoke (*Cynara cardunculus* var. *scolymus*) plants subjected to drought stress through hormonal regulation of source-sink relationship

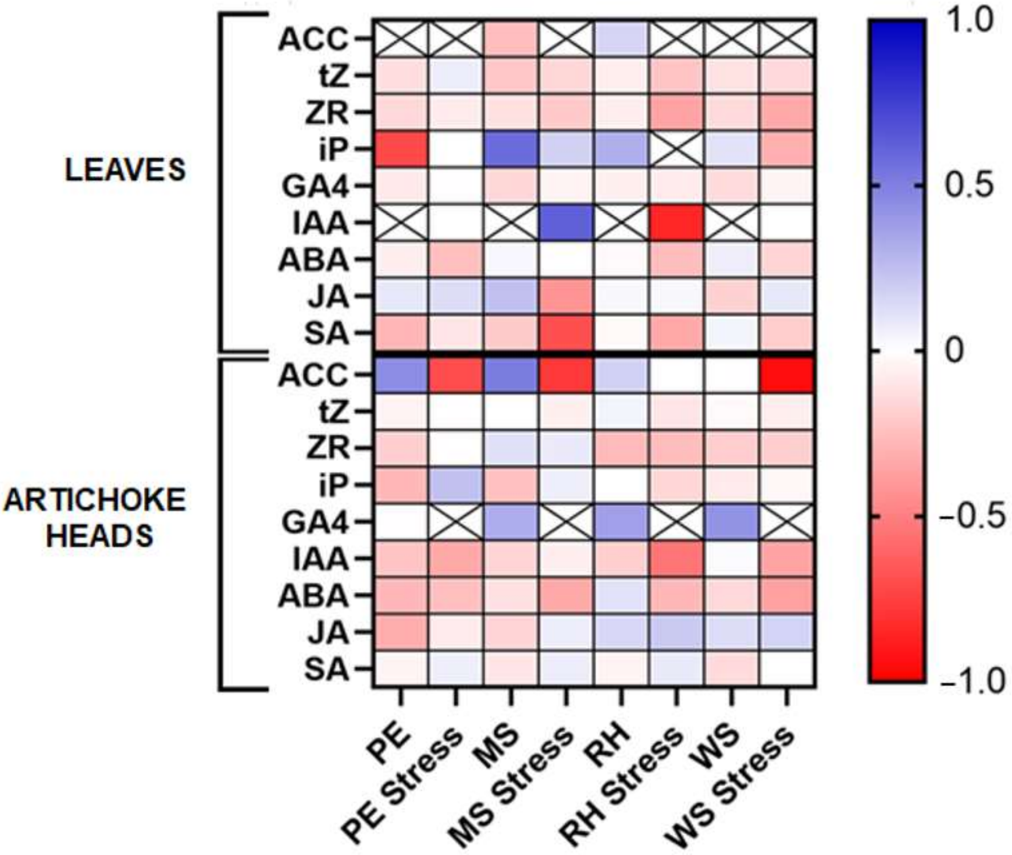

Figure S1. Cluster heat plot showing hormonal changes with respect to the bare soil treatment in leaves and heads of artichoke plants of the commercial variety Symphony non-mulched or subjected to different mulching treatments and cultivated under control (well-watered) and water stress (70% ETc) conditions. Abbreviations used: gibberellin A4 (GA_4), trans-zeatin (tZ), zeatin riboside (ZR), isopentenyladenine (iP), abscisic acid (ABA), 1-aminocyclopropane-1-carboxylic acid (ACC), indoleacetic acid (IAA), jasmonic acid (JA), salicylic acid (SA), polyethylene mulch (PE), mushroom substrate-based hydromulch (MS), rice hulls-based hydromulch (RH), and wheat straw-based hydromulch (WS).

CHAPTER V

The interaction between ecological hydromulching and arbuscular mycorrhizal fungi improves the growth and productivity of escarole (*Chichorium endivia* L.) by regulating nutrient use efficiency and hormonal balance

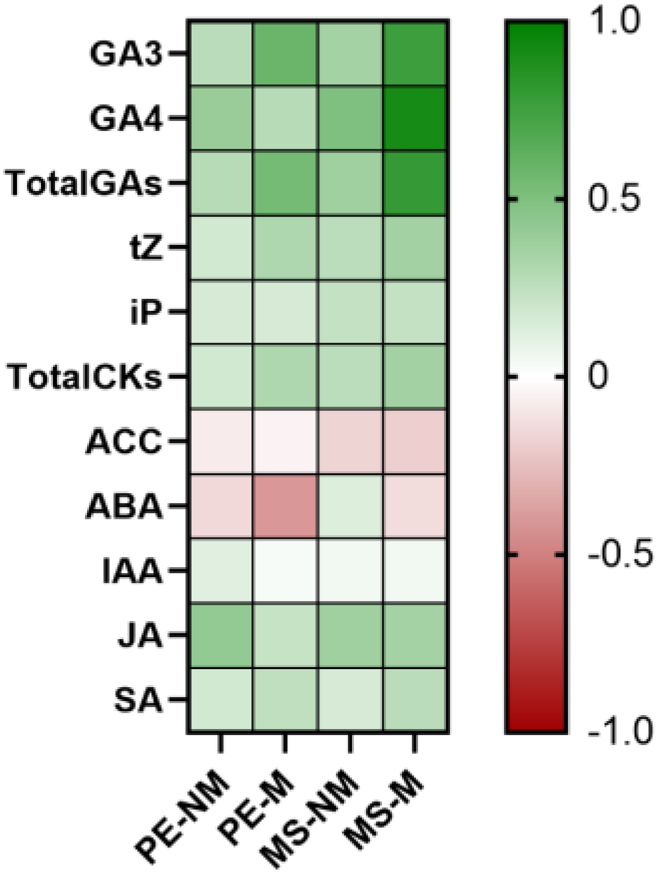

Figure S1. Cluster heatplot of the hormonal profile studied in escarole plants of the commercial variety "Symphony" non-mulched or subjected to different mulching treatments and cultivated under non-mycorrhized and mycorrhyzed conditions. Abbreviations used: Gibberellin A3 (GA_3), Gibberellin A4 (GA_4), total gibberellins (Total GAs), trans-zeatin (tZ), isopentenyladenine (iP), total cytokinin (Total CKs), 1-aminocyclopropane-1-carboxylic acid (ACC), abscisic acid (ABA), indole acetic acid (IAA), salicylic acid (SA), jasmonic acid (JA), polyethylene mulch (PE) and mushroom substrate-based hydromulch (MS).