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Effect of the substrate and the mycorrhiza application on the physiological functioning of ornamental species subjected to water and saline stress

Efecto del sustrato y la aplicación de micorrizas en el funcionamiento fisiológico de especies ornamentales sometidas a estrés hídrico y salino.

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Beatriz Lorente Pagán

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Pulido, L., **Lorente, B.**, Zugasti, I., Monteagudo, M., Bañón, S., Sánchez-Blanco, M.J., Ortuño, M.F. Tolerancia al riego con aguas no convencionales en Salvia officinalis y Asteriscus maritimus mediante la aplicación de herramientas biológicas para una mayor sostenibilidad. VII Jornadas Ibérico-Macaronésicas de Horticultura Ornamental. Tenerife, España. Septiembre 2018.

Zugasti, I., **Lorente, B.**, Ortuño, M.F., Hernández, J.A., Sánchez-Blanco, M.J. Efecto de *Pisolithus tinctorius* en el funcionamiento hídrico y fotosintético de plantas de *Cistus albidus* L. en condiciones de estrés hídrico y con aporte extra de nitrógeno. XIV Simposio Internacional Hispano-Portugués de Relaciones Hídricas en las Plantas. Madrid, España. Octubre 2018.

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RESUMEN
(SPANISH SUMMARY)

La investigación llevada a cabo en esta Tesis Doctoral aborda el efecto de diferentes estrategias de riego tanto en cuanto a las dosis de agua aplicadas, como en el origen y la calidad de las mismas en el cultivo de especies ornamentales con distintos fines medioambientales. Estas especies incluyen diferentes familias y se tratan de *Cistus albidus* L. (Cistaceae), *Asteriscus maritimus* L. (Asteraceae), *Salvia officinalis* L. (Lamiaceae), *Atriplex halimus* L. (Chenopodiaceae) y *Crithmum maritimum* L. (Apiaceae). Los ensayos realizados evalúan la respuesta y comportamiento de estas especies, en algunas de ellas, a un estrés hídrico severo y, en otras, al riego de aguas no convencionales, aguas regeneradas y salobres. Asimismo, también evalúan la posibilidad de utilizar sustratos alternativos a los comúnmente empleados para la producción de planta en vivero, que sean respetuosos con el medioambiente y con propiedades físico-químicas tolerables para las especies de cultivo, en distintas condiciones de riego. Se estudia como la composición del sustrato afecta el desarrollo del estrés hídrico y la posterior recuperación al inducir cambios fisiológicos en las plantas. A ello se le suma, la aplicación de hongos formadores de micorrizas (hongo arbuscular *Glomus iranicum* var. *tenuihypharum* y hongo ectomicorrícico *Pisolithus tinctorius*), herramientas biológicas, que pueden mejorar la eficiencia en el uso del agua y los nutrientes, mitigando los efectos negativos que las condiciones de estrés hídrico e iones tóxicos ocasionan en las plantas. Este trabajo de investigación se encuentra conformado por dos capítulos bien diferenciados, en el primero se avanza en el conocimiento sobre el aporte micorrízico en plantas de *Cistus albidus* que crece con diferentes tipos de sustrato y sometidas a estrés hídrico, y en el segundo se aborda las estrategias de idoneidad para el uso de recursos hídricos salinos para el riego en diferentes especies. Todos estos estudios están orientados a optimizar la eficiencia en el uso del agua de especies mediterráneas con fines ornamentales en la zona del Levante español, caracterizada por la baja calidad de los escasos recursos hídricos disponibles, obteniendo plantas de calidad, favoreciendo su adaptación a condiciones medioambientales desfavorables.

Capítulo 1.

Aporte micorrízico en plantas de Cistus que crecen con diferentes tipos de sustrato y están sometidas a estrés hídrico

The mycorrhizal contribution in Cistus plant growing with different types of substrate and submitted to water stress

Tradicionalmente, los residuos como los sólidos urbanos se han considerado sustratos no deseables. Sin embargo, algunos estudios han demostrado que, después del compostaje, estos residuos orgánicos pueden ser utilizados como medio de crecimiento con buenos resultados. El uso de compost como medio de cultivo puede ser adecuado para producir plantas en maceta, sin embargo, bajo déficit hídrico este sustrato puede ocasionar un estrés adicional para las plantas debido a sus propiedades físico-químicas, por contener iones fitotóxicos. El objetivo del capítulo 1.1 fue profundizar en los efectos de los elementos mayoritarios del sustrato compost (Al, Cu, Mn, P, Zn, Cr, B) en las relaciones de los distintos parámetros morfológicos, hídricos y nutritivos de plantas de *Cistus albidus*, así como en la velocidad y desarrollo de un estrés hídrico severo (supresión de riego) y la recuperación del mismo. Los ensayos se llevaron a cabo en una cámara de crecimiento con dos tipos de sustratos: un sustrato comercial compuesto por turba de sphagnum, fibra de coco y perlita (8: 7: 1) y un sustrato compost compuesto por una mezcla de abono en suspensión, fibra de coco y perlita (3: 6: 1). Un grupo de plantas se cultivaron en condiciones de buen riego, manteniendo valores de potencial hídrico foliar alrededor de -0,9 MPa (WW) y otro en condiciones de estrés hídrico (WS), donde se suprimió el riego durante un periodo de tiempo hasta alcanzar valores de potencial hídrico alrededor de -3,0 MPa, posteriormente, se restableció el agua en todas las plantas (período de recuperación). Desde el inicio del ensayo, los componentes del sustrato provocaron un efecto osmótico y/o tóxico. Aunque las plantas bien regadas mantuvieron un potencial hídrico foliar (Ψ_l) relativamente alto (-0,9 MPa) en ambos tipos de sustratos, la conductancia estomática (g_s) fue de 125 mmol m^{-2} s^{-1} en el sustrato comercial y 30 mmol m^{-2} s^{-1} en el compost. El tiempo necesario para alcanzar el valor umbral en el que se produce el estrés hídrico fue de 13 días en el sustrato comercial y 53 días en el compost. Las plantas bajo estrés hídrico en el sustrato comercial tuvieron valores significativamente más bajos de Ψ_l y g_s que las que estaban bien regadas, lo que no ocurrió en las plantas cultivadas en compost, las cuales mantuvieron valores de g_s similares en ambos tratamientos de riego (WW y WS) y acumularon menos biomasa que las que crecieron en sustrato comercial. El estrés hídrico en las plantas que crecieron en compost provocó un aumento de la epidermis adaxial, el parénquima y el mesófilo, mientras que en las que crecieron en sustrato comercial disminuyó las proporciones de los diferentes tejidos. Se encontraron valores más altos de peroxidación de lípidos en plantas cultivadas en ambos sustratos bajo estrés hídrico. El tiempo de recuperación de las plantas, hasta alcanzar valores de potencial hídrico en torno a -0,9 MPa, dependió del tipo de sustrato. La restauración del riego en sustrato comercial actúa como un nuevo estrés, reflejado en los mecanismos fotoquímicos.

En condiciones de estrés abiótico, la gestión del riego nos lleva a utilizar herramientas biológicas como la aplicación de micorrizas, lo cual puede mejorar la eficiencia en la absorción del agua y los nutrientes, obteniendo una planta de buena calidad. Pero ello está condicionado por distintos factores como el tipo de sustrato, medio de cultivo y especie de planta y hongo utilizado. En el capítulo 1.2 quisimos evaluar si el hongo *Glomus iranicum* var. *tenuihypharum*, que no se usa con frecuencia en el género Cistus, podría proporcionar beneficios, especialmente cuando las plantas están sometidas a estrés hídrico. El hongo utilizado quedó bien establecido en las raíces de las plantas de Cistus bien regadas en ambos sustratos (compost y comercial), sin embargo, el estrés hídrico impuesto obstaculizó la proliferación de micorrizas en el compost, lo que condujo a que las plantas cultivadas bajo estas condiciones tuvieran menor biomasa de hojas y raíces. Las plantas en compost acumularon mayores contenidos de Cl, K, Na, P y Zn en las hojas, la presencia de micorrizas redujo el contenido en Na y aumentó el fósforo, especialmente cuando el sustrato estaba bien regado. El estrés hídrico disminuyó el potencial hídrico de las hojas y las micorrizas mejoraron el estado hídrico de las plantas en ambos sustratos. El compost indujo ajuste osmótico foliar, menor intercambio de gases y menores valores de los parámetros fotoquímicos (Fv / Fm, Y (II). Las plantas micorrizadas tuvieron valores de Y (II) y qP más altos que las plantas no inoculadas. El compost disminuyó el contenido relativo de clorofila, pero en las plantas inoculadas estos valores aumentaron bajo estrés hídrico. Las plantas de *C. albidus* que crecieron en compost mantuvieron un buen equilibrio nutricional y una regulación osmótica eficiente. El tratamiento del estrés hídrico en este sustrato potenció el efecto osmótico, como lo reflejó la peroxidación lipídica y los valores de fotosíntesis. Se concluye que las plantas de *Cistus albidus* pueden completar su ciclo de crecimiento en cualquiera de los sustratos probados y que la inoculación con *Glomus iranicum* var. *tenuihypharum* fue compatible con ambos sustratos.

Las especies de Cistus pueden formar ectomicorrizas y hongos micorrízicos arbusculares que pueden traer beneficios cuando las plantas están bajo condiciones de estrés hídrico. Sin embargo, es menos conocido el efecto que tiene la aplicación de algunas ectomicorrizas sobre el comportamiento fisiológico y la regulación hormonal de las plantas en condiciones adversas, como el estrés hídrico, lo que dependerá de la extensión y duración del estrés. En el capítulo 1.3 quisimos estudiar la capacidad que tienen las plantas de *Cistus albidus* de hacer frente a un estrés hídrico severo (por supresión de riego), con la ayuda del hongo ectomicorrícico *Pisolithus tinctorius*, que posee una gran capacidad de micorrización y proporciona una mayor eficiencia del uso del agua y del aporte de nutrientes a la planta. El experimento se realizó durante tres meses en una cámara de crecimiento con plantas de *Cistus albidus* cultivadas en sustrato comercial a las que se aplicó el efecto combinado de la inoculación de *P. tinctorius* y dos tratamientos de riego (plantas bien regadas y bajo estrés hídrico). La ausencia de riego provocó una disminución significativa en el crecimiento aéreo de las plantas y tendió a disminuir el potencial hídrico del suelo en la superficie de la raíz, lo que condujo a una disminución del potencial hídrico foliar. En estas condiciones, el contenido de ácido abscísico y ácido salicílico aumentó mientras que el precursor del etileno disminuyó. Aunque los porcentajes de micorrización no fueron altos, la inoculación de *P. tinctorious* mejoró el estado hídrico y amortiguó levemente el aumento de la temperatura de las hojas de las plantas con estrés hídrico. La ectomicorriza disminuyó los valores de escopoletina en hojas de plantas sometidas a estrés hídrico, lo que indicó que las plantas inoculadas habían sido capaces de sintetizar mecanismos de defensa. Por tanto, *Pisolithus tinctorious* alivió algunos de los efectos nocivos de la escasez de agua en las plantas de Cistus, siendo su uso una opción sostenible en proyectos de jardinería o restauración.

Capítulo 2.

Estrategias de idoneidad para el uso de recursos hídricos salinos para el riego en diferentes especies

Suitability strategies for the use of saline water resources for irrigation in different species

A fin de paliar los efectos de la escasez de agua, se plantea el uso de recursos hídricos no convencionales, por ello, cada vez es más frecuente el uso de aguas residuales depuradas y aguas procedentes de desaladoras en agricultura en zonas de escasez. Sin embargo, su aplicación mal gestionada puede provocar cambios nutricionales, toxicidad iónica y alteración de compuestos que afectarán negativamente al cultivo. Estos efectos dañinos pueden mitigarse mediante el uso de micorrizas; sin embargo, la caracterización fisiológica de las especies más apropiadas para el uso de este tipo aguas es relevante para su uso en proyectos de paisajismo y jardinería. En el capítulo 2.1 quisimos identificar cuál de dos especies con diferente tolerancia a la salinidad, *Salvia officinalis* L. y *Asteriscus maritimus* L., eran más aptas para riego con aguas salobres, procedentes de aguas residuales tratadas (CE: 3,4 – 4 dS m^{-1}), así como el efecto de la micorriza arbuscular *Glomus iranicum* utilizando este tipo de agua. Además, se quiso evaluar la capacidad de recuperación de los parámetros fisiológicos de las plantas que habían sido regadas con aguas residuales tratadas, tras un período de riego con aguas de buena calidad. Para ello, estudiamos la respuesta morfológica y fisiológica de dichas plantas. El ensayo se llevó a cabo en una cámara de crecimiento y se dividió en tres fases: una primera fase, en la que tanto las especies inoculadas como no inoculadas se regaron con agua de buena calidad; una segunda fase, en la que la mitad de las plantas se regaron con aguas regenerada salinas; y una tercera fase, en la que las plantas fueron regadas nuevamente con agua de buena calidad (recuperación). Los niveles más altos de Na^+ y Cl^- medidos en el sustrato en Asteriscus no resultaron en un aumento de iones en la parte aérea de la planta, lo que tuvo menos efectos negativos en el crecimiento que en Salvia. La salinidad provocó una reducción en el potencial hídrico foliar, en la conductancia estomática y en la fotosíntesis neta de ambas especies. El porcentaje de micorrización fue mayor en Asteriscus que en Salvia, lo que mitigó la disminución del potencial hídrico foliar debido a las sales presentes en el agua. En las plantas de Asteriscus, las micorrizas suscitaron el cierre de los estomas, mientras que en Salvia los niveles de intercambio gaseoso fueron similares entre tratamientos. El ajuste osmótico se realizó en Salvia, aunque el contenido de prolina aumentó en ambas especies. Los daños producidos por las sales son más claros en Salvia, donde los valores de peroxidación lipídica fueron mayores. Así mismo, la apariencia visual de las hojas mostró síntomas de toxicidad en esta especie, aunque las micorrizas disminuyeron estos efectos. Aunque el contenido de Na^+ y Cl^- fue mayor en la fase de recuperación, el riego con agua de buena calidad indujo la recuperación de la peroxidación lipídica en ambas especies, así como la aparición de hojas nuevas en Salvia.

Las especies halófitas son capaces de hacer frente a un exceso de NaCl en sus tejidos, aunque algunas especies pueden diferir en su grado de tolerancia a la sal. Además, no está claro si pueden tolerar otros componentes o impurezas asociados con el uso de aguas no convencionales para el riego de dichas plantas. El experimento expuesto en el capítulo 2.2, se llevó a cabo en un invernadero con plantas de *Crithmum maritimum y Atriplex halimus*, plantadas en suelo. Dichas plantas se regaron con dos tipos de agua diferentes: aguas residuales regeneradas (RWW) (CE: 0,8-1,2 dS m^{-1}) y salmuera originada por ósmosis inversa (ROB) (CE: 4,7–7,9 dS m^{-1}). Ambas especies mostraron respuestas fisiológicas y nutricionales diferentes cuando se regaron con ROB. Las plantas Atriplex redujeron el potencial hídrico foliar y mantuvieron la turgencia de las hojas como consecuencia de un proceso de ajuste osmótico. Atriplex mostró una eficiencia intrínseca en el uso del agua mayor que Crithmum, independientemente del tipo de agua utilizada. El estado hídrico y la eficiencia fotosintética de Crithmum fueron similares en ambos tratamientos. Crithmum presentó mayor acumulación foliar de iones B y Ca, mientras que Atriplex presentó mayor cantidad de K, Mg, Na y Zn. Las plantas de Crithmum regadas con ROB presentaron mayores concentraciones de ácido 1-aminociclopropano-1-carboxílico y trans-zeatina-glucósido, mientras que la concentración de ácido abscísico fue menor. Atriplex mostró una menor concentración de trans-zeatina-ribósido y escopoletina. Las características asociadas al agua de riego no influyeron negativamente en el desarrollo de ninguna de estas especies, lo que confirma el uso de salmuera como alternativa para riego de estas especies.

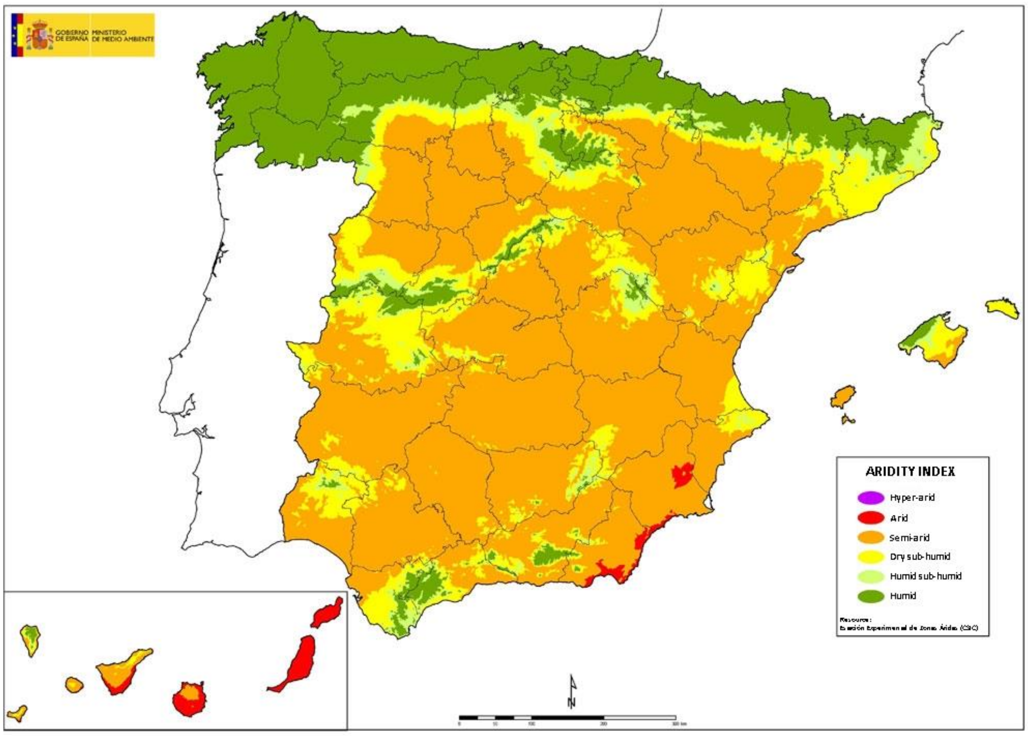
BACKGROUND

1. Climate and vegetation of the Mediterranean area

In Spain there are three major biogeographic regions: The Eurosiberian, the Mediterranean and the Macaronesian, each one of this region has characteristic soils, which closely depend on the type of rock, the climate, the vegetation, as well as the time elapsed since its formation and the incidence of human activities. The soil also exerts its own influence, since it is the sustenance of the vegetation, the reservoir of water and nutrients that the plants use and, in addition, it plays an important hydrological regulatory role. But both the soils and the vegetation have suffered an intense alteration by human activity that, for centuries, has developed in Spain. For this reason, the extension of the vegetation corresponding to these biogeographic regions has been reduced and, in addition, their floristic composition has been modified.

The Mediterranean region occupies 80% of the Peninsula and the Balearics, which is characterized by mild winters and hot and dry summers, conditions that subject the vegetation to considerable water stress.

When the result between annual precipitation and potential evapotranspiration is between 0.05 and 0.65, we speak about susceptible to desertification areas, and these are arid, semi-arid and dry sub-humid. The combination of factors and processes such as aridity, drought, erosion, forest fires, overexploitation of aquifers, etc., gives rise to different landscapes or typical scenarios of desertification in Spain.



Picture 1. Aridity map, the colors represent the degree of aridity from highest to lowest: purple (hyper-arid), red (arid), orange (semi-arid), yellow (dry sub-humid), light green (humid sub-humid) and dark green (humid).

2. Effects of water deficit and mechanisms of resistance to drought in plants

Plants develop mechanisms to cope with water deficit (Nilsen and Orcutt, 1996). They are capable of developing morphological, physiological and biochemical modifications that allow them, on the one hand, to avoid water stress and, on the other, to tolerate it. Thus, some plants synthesize solutes in their cells (osmotic adjustment), which help them retain water, so they can grow faster. Other species respond to water deficit by developing mechanisms in order to regulate transpiration, such as stomatal closure, reduction of leaf area and epinasty (Sánchez-Blanco et al. 2002).

Depending on the water needs, plants can be classified into three groups: hydrophytic, when they can live totally or partially submerged in water (maximum leaf water potential, Ψ_l: -5 to -10 bars); mesophytic, when they receive a little contribution of water (Leaf water potential, Ψ_l: around -20 bars) and xerophytic, if they are adapted to arid environments (Leaf water potential, Ψ_l: more than -40 bars) (Nilsen and Orcutt, 1996).

Currently, there is a marked tendency to carry out projects in gardening, revegetation and landscaping of semi-arid areas, which respond to sustainable designs of biodiversity, use of ornamental species adapted to this environment and saving water resources. The strategies to carry out these projects, in order to avoid or alleviate the negative effect of frequent abiotic stresses during the implantation and maintenance processes of the plants, could be the appropriate choice of plant material, production methods and nursery preconditioning, to obtain seedlings that adequately tolerate transplantation. There are many species potentially suitable to use in landscaping projects in the Mediterranean region, as the species tested in this study: *Salvia officinalis, Cistus albidus, Atriplex halimus, Crithmum maritimum and Asteriscus maritimus.*

3. Use of alternative substrates for nursery plant production

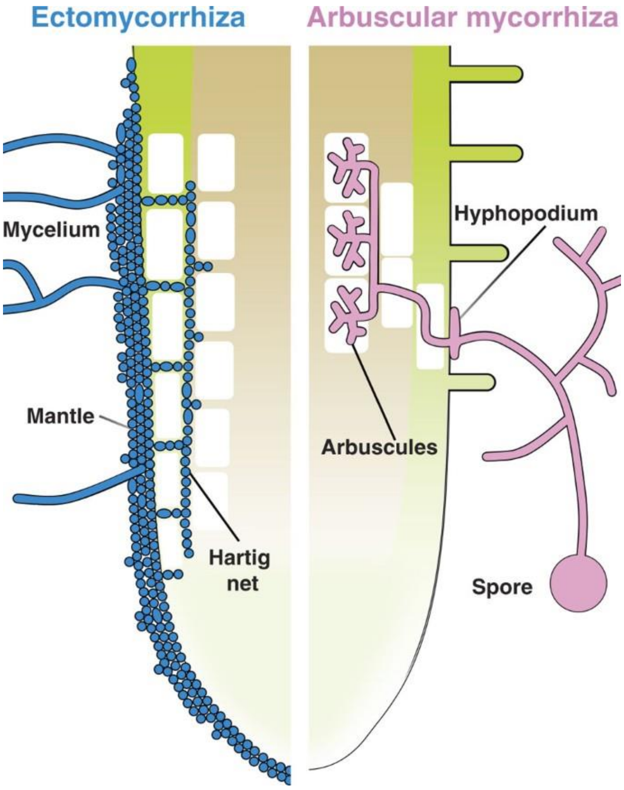
Peat and natural soils have been used frequently as substrates in nurseries for the production of ornamental plants (Pagés and Matallana, 1984). However, the strong dependence on imported materials, its high price and the consideration of peat as a non-renewable resource has stimulated the use of alternative materials as plant growth substrates (Abad et al., 2001), which can be respectful with the environment and have optimal physical-chemical properties for the plant. Since ever, municipal solid waste and even green waste have always been considered unsuitable substrates. However, some studies have shown that these are good growing media, replacing peat, after composting (García-Gómez et al., 2002). Although in landscaping the small variations in the particles size and their mineral composition are not so important, this together with other factors, can have a significant importance in the water and nutritional behaviour, development and quality of the plant when a limited volume is used, as in the case of the use of small containers like pots (Larcher and Scariot, 2009).

Furthermore, these substrates usually present some phytotoxic ions and heavy metals that can alter characteristics of the plants, such as the decrease in leaf area, in order to avoid water loss through the leaves (Barceló and Poschenrieder, 1990; Perfus-Barbeoch et al., 2002). These toxic elements can also modify the metabolic activities in leaves and chloroplasts, due to the alteration that they produce in the electron transport chain (Qadir et al., 2004). In some studies, the metals present in the substrate have also altered the permeability of the root membranes, modifying the water absorption and the hydric state of the plant (Przedpelska-Wasowicz and Wierzbicka, 2011).

For all these reasons, the compost to be used as a component of substrates must have specific physical and chemical characteristics to ensure adequate support and, at the same time, act as a reservoir for water and nutrients, without excess toxicity or pathogenic microorganisms (Raviv et al., 1986; Larcher and Scariot, 2009).

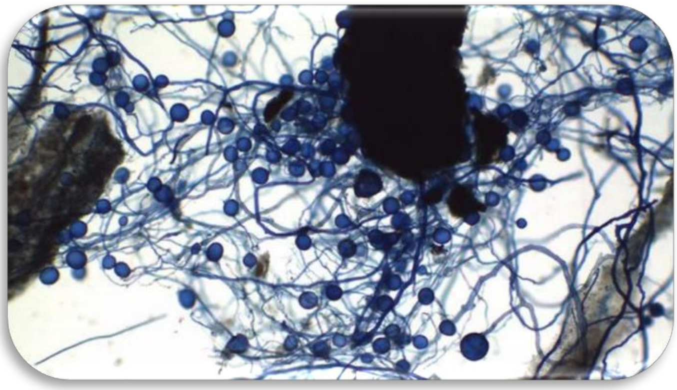
4. Application of mycorrhizal fungi on plants under abiotic stress conditions

Soil microorganisms have been widely shown to interact with plant roots and soil constituents. However, most microbiological rhizosphere studies have focused on bacteria and fungi. Mycorrhizae are symbiotic associations between plant roots and fungi. Plants can form both, ectomycorrhizae (ECM) and arbuscular mycorrhizal fungi (AMF), most plants form a single type of association, although there are dualistic species such as Cistus, Populus, Salix and Eucalyptus (Smith and Read, 2008; Comandini et al., 2006), which can associate to both types of fungus. Arbuscular mycorrhizal fungi are the most common mycorrhizal associations, which is estimated to occur in approximately two-thirds of terrestrial vascular species (Smith and Read, 2008). Several studies have shown that inoculation with these fungi has a greater positive effect when the plants are subjected to some type of stress, either biotic (such as the attack of pathogens) or abiotic (drought, salinity, heavy metals, organic pollutants, etc.) (Miransari et al., 2011). However, the response of the plant will depend on both the species used and the fungus inoculated, and the duration and stress to which they are subjected (Ruíz-Lozano et al., 1995; Mardukhi et al., 2011).



Picture 2. Mycorrhizal association scheme depending on the type of fungus: Ectomycorrhiza (left) and arbuscular mycorrhiza fungus (right).

Glomus iranicum var. *tenuihypharum* is an arbuscular mycorrhizal fungus that, compared to others, promotes an extensive extramatric mycelium causing the efficient transfer of nutrients from the soil to the plant. Sporulation occurs outside the root, promoting a more efficient symbiosis and achieving higher colonization rates at the expense of lower carbohydrate consumption. It tolerates high amounts of salts, so it is not necessary to reduce plant fertilization. It also improves the soil structure and stimulates the microbial population of the rhizosphere (Błaszkowski et al., 2010).



Picture 3. Spores and hyphae of *Glomus iranicum* var. *tenuihypharum* (property of Symborg).

Pisolithus tinctorius is an ectomicorrhyza that measures between 5 and 20 cm in height and up to 10 cm in diameter. It has yellowish-brown fibrous, cinnamon-brown spores, 7 to 12 µm globose and spines up to 2 µm long (Gómez-Romero et al., 2015). It is one of the most widespread ECM fungi and can establish associations with most species. It can survive both in poor soils and with high amounts of heavy metals, and it is also capable of withstanding large periods of drought stress (García-Rodríguez et al., 2006; Alguacil et al., 2003).

Picture 4. a) Image of the fungus *Pisolithus tinctorius* and b) mycorrhizal association (property of Aurelio Fierros).

At the nursery field, the purpose of controlled inoculation aims to achieve reduce mortality in plantations, minimizing the critical phase of transplantation. Another objective is the defence against possible soil pathogens, alleviating the harmful effects and the recovery of contaminated and degraded soils (Honrubia et al., 1992). That is why inoculation with mycorrhizal fungi has been carried out in the nursery to species that have been destined for repopulation (Valdés-Ramírez et al., 2010).

5. Use of saline water resources for irrigation

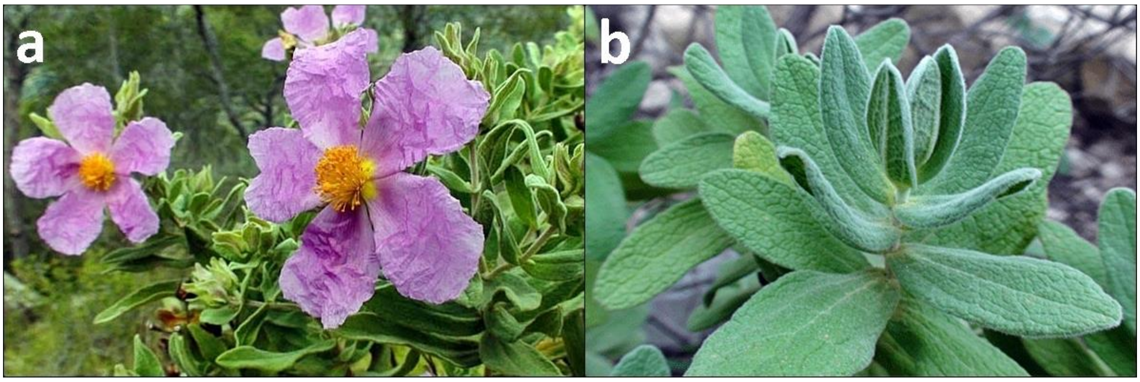
The Region of Murcia has a semi-arid climate, where the demand for water is increasing, so it is necessary to propose alternatives to the use of fresh water, one of them would be the use of reclaimed water (RW) (Mujeriego, 1990; Anderson et al., 2001; Asano, 2006; Grattan et al., 2015). Among the advantages of the use of reclaimed water is the possibility that agriculture offers in reducing environmental pollution, since the direct discharge of these waters into the natural channel would require a more expensive treatment to minimize contributions such as macronutrients or organic substances that are difficult to mineralize, which, however, can be positive for crops when used as fertilizer (Asano and Levine, 1996; Stott et al., 1997; Levine and Asano, 2004). This way, RW might contain an important load of nutrients easily accessible to plants; nitrogen, phosphorous and potassium (Aziz and Farissi, 2014). It has been shown that RW can have a positive effect as long as the accumulation does not exceed the toxicity threshold (Ofori et al., 2021). Moreover, some authors estimate an increase of 59% in the accumulation of organic matter with the use of these regenerated waters in irrigation (Pedrero and Alarcón, 2009). Therefore, an appropriated use of this type of water might promote plant growth and consequently improve yield, while saving fertilizer costs.

However, they also usually have high salts concentrations in addition to other chemical contaminants, such as inorganic compounds, heavy metals, pathogens and many other complex compounds, that may affect negatively growth, development, productivity and quality of plants by osmotic, toxic and nutritional effect, in addition to oxidative stress (Quist et al., 1999; Gori et al, 2000; Hernández et al 2001; Flowers et al., 2010; Gómez-Bellot et al., 2015; Intriago et al., 2018).

6. Ornamental species studied

a. *Cistus albidus*

Cistus albidus L., commonly known as white-leaved Rockrose or white steppe, is a woody shrub belonging to the Cistaceae family, with a grayish-green color that can measure from 40 to 150 cm in height. Its branches can be erect or ascending, with opposite leaves, pale gray, densely tomentose, sessile, flat and revolute; ovate oblong, 1.5-6.5 x 1-2.5 cm, with very prominent veins on the underside. They have flowers in terminal umbelliform cymes, with 5 wrinkled and drooping petals, from pink to purple, and numerous stamens. The fruits are a 6 to 12 mm hairy capsule, open by means of 5 valves (Alcaraz et al., 1998a).

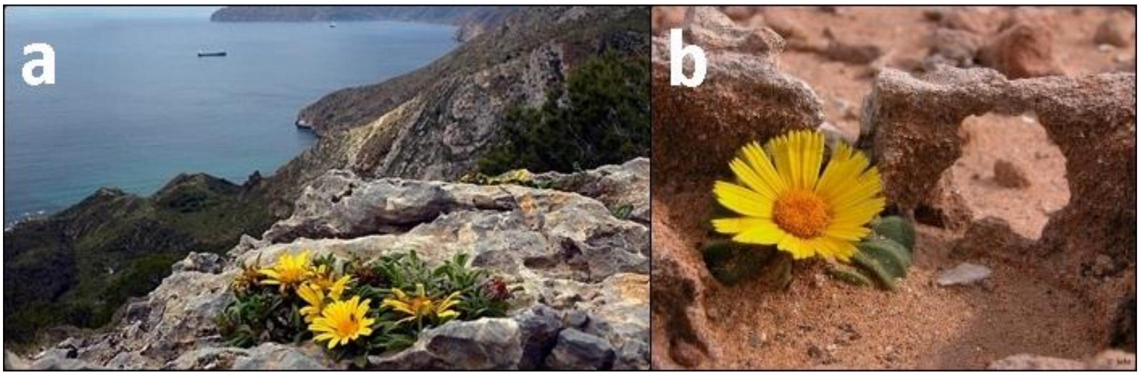


Picture 5. Detail of a) flowers and b) leaves of *Cistus albidus*.

Martín Bolaños and Guinea (1949) indicated that this plant was typical of the western Mediterranean region. As they describe, it was found in Spain both in clay and limestone soils, in bushes, hills, as well as in low-lying regions and mountain areas. Castroviejo et al. (1993) place the *Cistus albidus* in the bushes that correspond to the regressive stage of Mediterranean forests, in regions near the sea or in areas with a dry climate, with little cold in winter and very hot in summer.

b. *Asteriscus maritimus*

Asteriscus maritimus L. Greuter is commonly known as sea daisy, starfish or asterisk. It is a small perennial herbaceous plant belonging to the Asteraceae family, adapted to semi-arid areas. It is a creeping plant 2-20 cm high, rough, more or less erect, with a woody base, often with many branches, bushy and covering large areas. It has alternate leaves, petiolate, lanceolate-spatulate, 3 x 1 cm, uninervated and hairy. It has numerous flowers, grouped in chapters (Bärtels, 2003); lanceolate bracts, green; two types of flowers, florets on the inside and ligules on the outside, yellow, with elongated outer petals, with three "teeth" at the apex. Achene type fruit. It blooms almost all year. It reproduces by seeds or cuttings in summer. Due to its morphology, it is considered an excellent species as a groundcover to cover large areas in combination with other species (Alvarado et al., 2009).



Picture 6. *Asteriscus maritimus* plants (image b property of José Antonio López Espinosa).

It occurs mainly in rocky areas near the coast, in sandy and scrubland areas, in warm/semi-arid environments. It supports high levels of insolation, droughts and saline soils (halophyte), making it an interesting plant to use in the restoration of saline environments (Estrada et al., 2013).

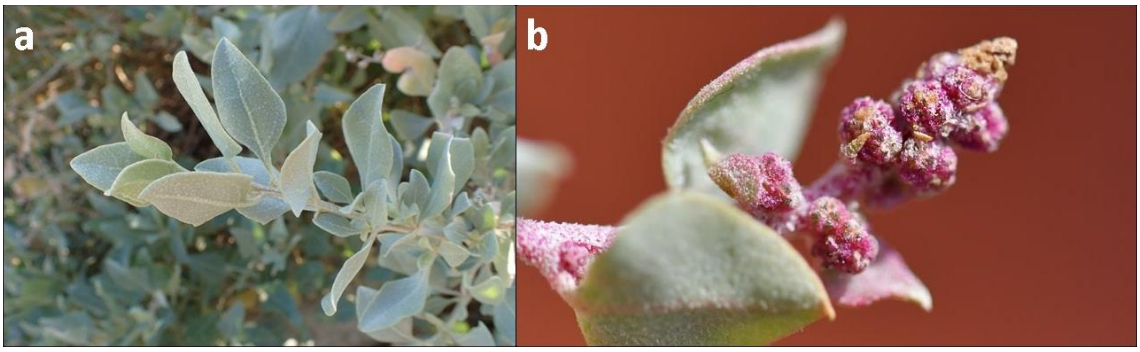
c. *Salvia officinalis*

Salvia officinalis L. is an aromatic shrub up to 70 cm high, of the Lamiaceae family. It has opposite, elliptical and hirsute leaves, with well-marked veins. During flowering, flowers appear in clusters of 5-10 flowers, bilabiated and with a purplish-blue color. Its natural distribution includes the western Mediterranean zone, particularly abundant in hot and arid zones, and on stony slopes, although it has been introduced and acclimatized in many other countries (Bärtels, 2003). This is due to its medicinal and culinary uses. Its diuretic, anti-inflammatory and astringent properties have been known since ancient times, and it can be used as a spice (Altindah and Altindal, 2016).

Picture 7. a) Leaves and b) flowers of *Salvia officinalis*.

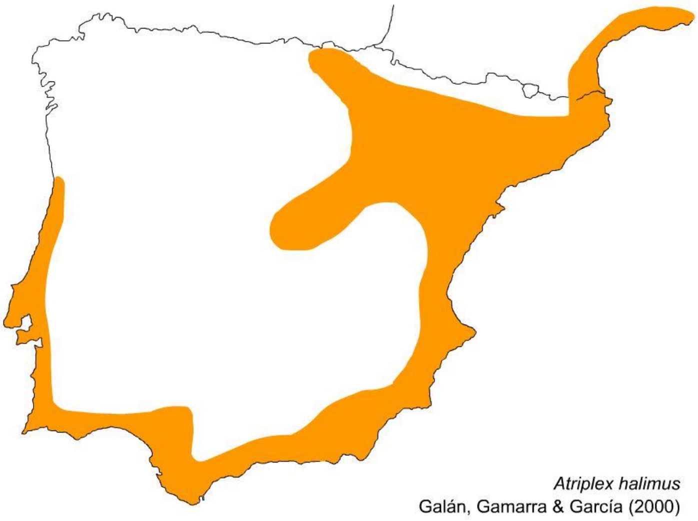
a. *Atriplex halimus*

Atriplex halimus L. commonly known as Mediterranean saltbush or orach, is a species of evergreen shrub in the Chenopodiaceae family. It can reach 2.5 m in height, branched from the base and with a silvery-white color. It develops a powerful root system capable of thriving at depths of 2 to 3 m (Cornelini et al., 2008), up to 10 m (Houèrou, 1992). Alternate leaves with short petioles, polymorphic (Castroviejo et al., 1990) and with trichomes on the upper and backside, with the main veins marked on the backside and up to four centimeters in length. It is a monoecious plant and has little showy terminal inflorescences. Achene type fruit and its fall is facilitated by wind, rain or the passage of animals (Ruiz de la Torre et al., 1996).



Picture 8. a) Leaves and b) fruits of *Atriplex halimus*.

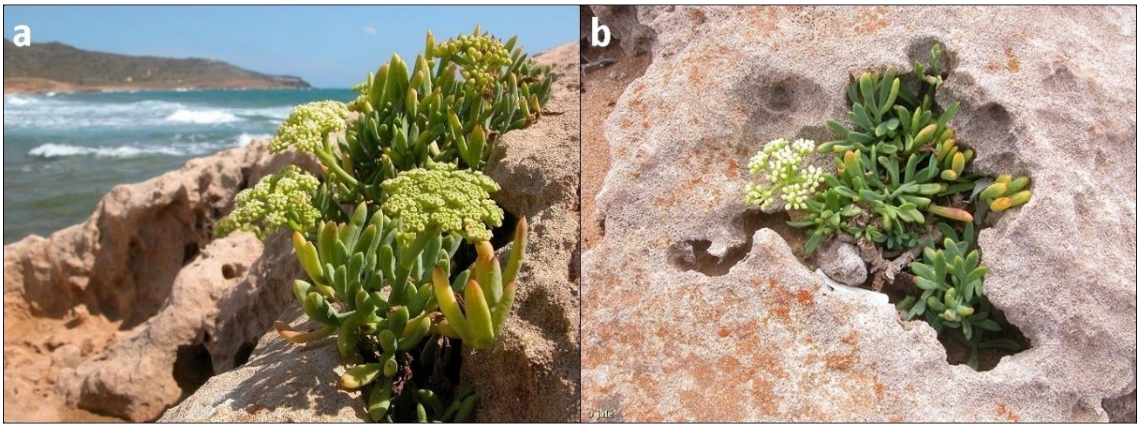
It is usually found in areas with high solar radiation and sandy soils. Withstands continuous exposure to wind and is also resistant to frost down to -10ºC.



Picture 9. Location of *Atriplex halimus* in the Iberian Peninsula.

d. *Crithmum maritimum*

Crithmum maritimum L. commonly known as sea fennel or marine parsley, is a halophytic, perennial and fleshy plant, belonging to the Apiaceae family. Its stems can reach 15 to 45 cm in height. It has fleshy leaves (20-50 x 3-5 mm) and sublobulated, linear lanceolate or acute with a rounded outline. Its flowers are arranged forming an umbel, very numerous, small, not very showy and, generally, they are hermaphroditic. The fruits measure 3-6 x 1.5-3.5 mm and are smooth, ovoid or oblong. It is an edible plant found on the Mediterranean coast that wild grows. They tend to grow on cliffs and beaches, as well as in rock crevices, where their roots go deep. We rarely find it far from the sea. In addition to salinity, it is a very resistant plant to drought and is capable of flowering in summer.



Picture 10. *Crithmum maritimum* plants (images owned by José Antonio López Espinosa).

CHAPTER 1

The mycorrhizal contribution in Cistus plant growing with different types of substrate and submitted to deficit irrigation

Chapter 1.1

Substrate composition affects the development of water stress and subsequent recovery by inducing physiological changes in *Cistus albidus* plants

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Abstract

Organic residues (compost) can be used as growth medium but may contain phytotoxic ions that, combined with a water deficit may alter the behavior of plants. The experiment was carried out in a growth chamber with *Cistus albidus* in a commercial substrate, C (sphagnum peat, coconut fiber and perlite, 8:7:1) and a mixture of compost substrates, Cp (slurry compost, coconut fiber and perlite, 3:6:1). Plants were grown in pots under well-watered, maintaining values of Ψ_l around -0.9 MPa (WW) and water-stressed (WS) conditions, where the irrigation was removed until reached values of Ψ_l around -3.0 MPa (water stress period), after then, water was re-established in all plants (recovery period). Although, the well-watered plants had a leaf water potential (Ψ_l) around -0.9 MPa, stomatal conductance (g_s) was 125 mmol $m^{-2}s^{-1}$ in the commercial substrate and 30 mmol $m^{-2}s^{-1}$ in compost. The time taken to reach the threshold value at which water stress occurs was 13 days in the commercial substrate and 53 days in compost. Water-stressed plants in the commercial substrate had significantly lower values of Ψ_l and g_s than well-watered. Plants in compost maintained values of g_s similar in both irrigation treatments (WW and WS) and accumulated less biomass than those that grown in commercial. The water stress in compost led an increase in the adaxial epidermis, parenchyma and mesophyll, whereas water stress in commercial the proportions of the different tissues decreased. Higher lipid peroxidation values were found in plants grown in both substrates under water stress. The recovery time of the plants, until manage Ψ_l values around -0.9 MPa, depended on the type of substrate. The restoration of irrigation in commercial substrate act as a new stress, as reflected in the photochemical mechanisms.

Keywords: substrate, osmotic stress, mineral content, water relations, lipid peroxidation, chlorophyll fluorescence.

1. Introduction

Nurseries that produce ornamental plants frequently used organic substrate made with Sphagnum peat (Blievernicht et al. 2013). However, it is a non-renewable resource and, in southern Europe at least, peat needs to be imported, and, as its cost has risen and its properties become more variable, the search for alternative materials has intensified (Abad et al., 2001; Ribeiro et al., 2007). Recently, several formulations of alternative materials for potting ornamental plants have been developed, particularly for use with herbaceous and annual species. The effect of adding composted waste to a peat as growth medium is both economically and environmentally attractive (Hargreaves et al., 2008). Some studies have even shown that, after composting, these organic residues can be used as a culture medium instead of peat with very good results (García-Gómez et al., 2002). Nevertheless, the use of alternative growing media requires an assessment of their physical and chemical properties, which must be able to provide adequate support and act as a reservoir of water and nutrients (Larcher and Scariot., 2009). Also, in all such studies, there is a need to quantify the toxic effects of any metals in the substrate.

Certain phytotoxic ions and heavy metals present in these organic residues can affect the morphological and anatomical characteristics of plants, such as leaf area reduction, which influence loss of water through the leaves (Barceló and Poschenrieder, 1990; Poschenrieder and Barceló, 2004; Perfus-Barbeoch et al., 2002). Substrate composition may also affect root membrane permeability to water (Przedpelska-Wasowicz and Wierzbicka, 2011), some studies have pointed to alterations in water absorption and the water status of the plant under an excess of both essential and non-essential metals (Perfus-Barbeoch et al., 2002; Rucinska-Sobkowiak., 2016). In addition, toxic elements may also alter the metabolic activities of several tissues and plant organelles, particularly in leaves and chloroplasts, modifying the structure and function of photosystem II due to perturbations in the electron transport chain (Qadir et al., 2004).

For all the above, any compost used as a component of a substrate must have specific physical characteristics that will provide it with good aeration and water holding capacity, but also be free of excessive phytotoxicity and pathogenic microorganisms (Raviv et al., 1986).

On the other hand, the growing concerns about water scarcity have focused more attention into possible mechanisms for enhancing water use efficiency (WUE) and drought resistance, which according to Zhang et al. (2002) is regulated by genetic and environmental factors and by cultivation methods.

Various factors that contribute to photosynthetic performance under stress conditions have been studied, including osmotic adjustment, anatomical structure, aging conditions (Xu et al., 2010; Yuping et al., 2017), although their relationship with stomatal characteristics is still not entirely established. Moreover, it is well known that a plant's response to water stress depends not only on the intensity of any deficit treatment, but also on the time of exposure to the stress (Álvarez and Sánchez-Blanco, 2013; Álvarez et al., 2018).

In the case of *Cistus albidus*, a typical Mediterranean shrub, plants respond to water deficit by developing avoidance mechanisms based on the limitation of plant growth, a reduction in leaf area and epinasty, which can be considered complementary mechanisms for the regulation of transpiration and the amount of intercepted radiation (Sánchez-Blanco et al., 2002; Munné-Bosch et al., 2002).

However, knowledge of the relationship between tolerance to drought and the characteristics of the culture medium, as well as information regarding the response of plants during water stress recovery, is scarce, and the physiological mechanisms involved in the recovery process remain poorly understood (Chaves et al., 2011).

The objective of this work was to evaluate how compost with a high Zn, Mn, Al and Cu content and a high water retention capacity can modify the development and the speed of an imposed water stress when used as substrate, as well the resistance to water stress and subsequent recovery of *C. albidus* plants. For this, changes in biomass, water relations, gas exchange, photochemical efficiency, lipid peroxidation, nutrients and leaf anatomical structure were evaluated. The importance of studying the capacity for recovery following water stress relief was also taken into account.

2. Material and Methods

2.1. Experimental conditions and treatments

Picture 1. *Cistus albidus* L. plants in the growth chamber.

Fifty single rooted cuttings (25- to 30-cm tall) of *Cistus albidus* L. plants were grown in 14x12x12 cm pots, of which 25 were filled with Sphagnum peat, coconut fiber and perlite (8:7:1) as commercial substrate (C) and 25 were filled with a mixture of slurry compost, coconut fiber and perlite (3:6:1) as a compost substrate (Cp). Both substrates were supplemented with Osmocote Plus (14:13:13 N, P, K + microelements). The experiment was carried out in a controlled growth chamber, under the following conditions: natural temperature, 23 ºC/18 ºC (day/night); Photosynthetic photon flux density, 350 μmol $m^{-2}s^{-1}$; Photoperiod, 16/8 h (light /dark) and relative humidity of 60%. Once *C. albidus* was adapted to the chamber conditions, the irrigation treatments consisted in well-watered plants (WW), maintaining values of Ψ_l at light hours around -0.9 MPa (CWW and CpWW), and then water-stressed (WS) where the irrigation was removed to half of the plants until them reached values of Ψ_l around -3.0 MPa (CWS and CpWS), considered as a several drought stress (water stress period). After then, water was re-established on the plants which had suffer drought until manage Ψ_l values around -0.9 MPa again (recovery period). Plants were watered manually, using tap water whose electrical conductivity was 1.0 dS m^{-1}. Substrate's field capacity was calculated according Álvarez et al. (2013). Each pot with its plant was weighed before each irrigation event, and the volume of irrigation water required to refill the pot to its threshold level was calculated and added to each plant.

The duration of the experiment was 131 days. The stress phase began 58 days after acclimatization of the plants to the chamber, for CWS this phase was in effect for 13 days and 53 days for CpWS.

2.2. Substrate and leaf mineral content

The mineral content of leaf was analysed from five samples per treatment at the end of stress and recovery period. At the beginning of the experiment, three substrate samples per type of substrate were collected and analysed. The macronutrient concentrations were determined in a digestion extract with HNO3:HClO4 (2:1, v/v) using an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES IRIS INTREPID II XDL). The concentration of Cl was analysed by a chloride analyser (Chloride Analyser Model 926, Sherwood Scientific Ltd.) in the aqueous extracts obtained by mixing 100 mg of powdered dry sample with 40 mL of water before shaking for 30 min and filtering.

2.3. Growth and Survival

Growth was considered in relation to the height and dry weight of shoots and roots measured at the end of stress and recovery periods. After gently washing the substrate from the roots, five plants per treatment were individually separated into shoots (leaves and stem) and roots and the different organs were washed with distilled water. Then, leaves, stems and roots were oven-dried at 60 ºC until they reached constant weight in order to measure their respective dry weights (DW).

The survival rate was calculated by counting the number of plants of each treatment that had survived at the end of experimental period, dividing by the number of plants originally planted and multiplying by 100 to express survival as a percentage.

2.4. Water relations

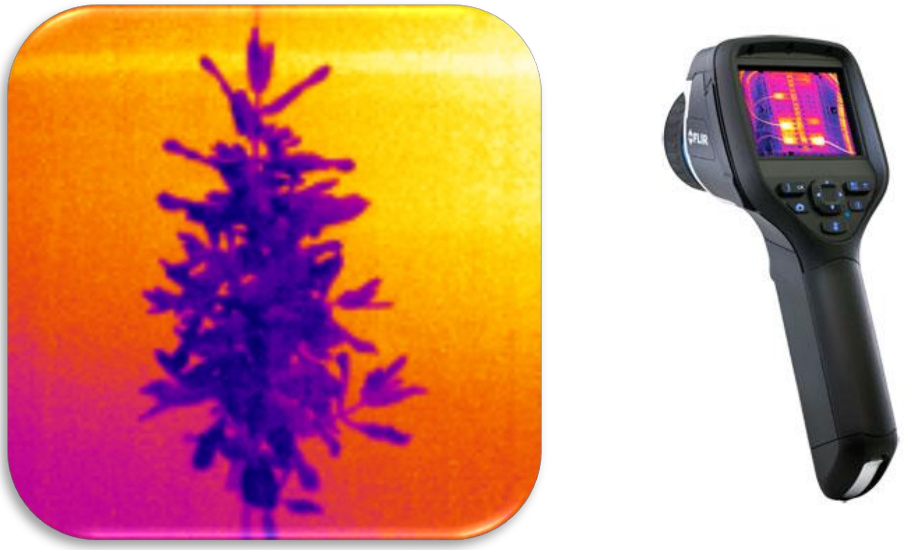
Changes throughout the experimental period in leaf water potential (Ψ_l), and osmotic water potential at full turgor (Ψ_{100s}) were measured in 5 plants per treatment. Ψ_l was determined according to the method described by Scholander el al. (1965), using a pressure chamber (Soil Moisture Equipment Co., Santa Bárbara, CA, USA, mod. 3000), while the leaves were pressurized at a rate of 0.03 MPa s^{-1}. The osmotic water potential at full turgor (Ψ_{100s}) was also estimated using excised leaves with their petioles placed in distilled water overnight to reach full saturation. Then, these leaves were frozen in liquid nitrogen (-196 ºC) and stored at - 30 ºC. After thawing, Ψ_{100s} was measured in a drop of the extracted sap using a filter paper disc placed in a C-52 and using a Wescor 5520 vapor pressure osmometer (Wescor Inc., Logan, UT, USA).

2.5. Gas exchange and leaf temperature

The net photosynthesis rate (P_n) and stomatal conductance (g_s) were determined in 5 plants per treatment using a portable gas exchange device, LICOR LI-6400 (LI-COR Inc., Lincoln, NE, USA). The flow rate of circulating air within the system was approximately 300 µmol s^{-1}, with a sheet vapour pressure deficit to air of about 2 KPa.

The CO_2 concentration was fixed at 400 ppm and the photosynthetically active radiation (PAR) at 600 µmol $m^{-2}s^{-1}$. The measurements were carried out throughout the experimental period.

At the same time, leaf temperatures were measured by thermography, taking thermal images in four plants per treatment with a thermal camera (ThermaCam FLIR-e50 System, Inc., Danderyd, Sweden). The background temperature, distance of the camera from the canopy, air temperature, emissivity and relative humidity were used as input at the start of each series of measurements, permitting the camera to automatically correct for atmospheric transmission based on these data. Emissivity for leaf measurements was set at 0.96 (Leinonen et al., 2006). However, previously to the measurement set, background temperature was determined as the temperature of a crumpled sheet of aluminium foil in a similar position to the leaves of interest with the emissivity set at 1.0. The distance at which images were taken was 0.5 m. The images were processed with ThermaCam Researcher Professional 2.10 software (FLIR Quick Report).



Picture 2. *Cistus albidus* plant seen through thermal camera (left) and the thermal camera (right).

2.6. Chlorophyll fluorescence parameters

Three leaves per treatment were allowed to adapt the dark for 20 min (Acosta-Motos et al., 2015), in order to achieve maximum stomatal opening, and the maximum and minimum fluorescence values were measured with a fluorometer (IMAGING-PAM M-series, Heinz Walz, Effeltrich, Germany. Kinetic analyses were made with actinic light (81 μmol quanta m^{-2} s^{-1} PAR) on repeated pulses of saturating light at 27008 μmol quanta $m^{-2}s^{-1}$ PAR for 0.8 s at 20 s intervals (Clemente-Moreno et al., 2013). As regards the photochemical process, quantum yield (Y (II)), photochemical quenching (qP) and the efficiency of PSII (Fv/Fm) were measured. Non-photochemical quenching (qN) is related to heat dissipation and can be used as an indicator of stress due to its sensitivity (Maxwell and Johnson, 2000).

2.7. Lipid peroxidation

Following the method described by Acosta-Motos et al. (2015), the concentration of substances that react with thiobarbituric acid (TBARS) was estimated. For this, 0.1 g of leaves were homogenized in a 1M perchloric acid solution (w/v 1/10) and then centrifuged at 1400 g for 10 min, and 0.4 mL of supernatant was taken and mixed with 1.2 ml of 50% TBA. This mixture was incubated at 90 ºC for 20 min and the reaction was stopped by placing the Eppendorf on ice. Finally, after 5 min centrifugation at 10000 g, the absorbance was measured at 532 and 600 nm. The concentration of TBARS was calculated from the absorbance value given by the difference between the values obtained at 532 and 600 nm and using a molar extinction coefficient of 155 nM^{-1} cm^{-1}.

2.8. Light microscopy and morphometrical analysis

Leaf sections (1×1 mm^2) from the most recent fully expanded leaves from the central region of Cistus leaves were used for light microscopy analysis. These samples were fixed and postfixed according to Fernández-García et al. (2014). Semi-thin sections (0.5–0.7 µm) thick were cut with a Leica EM UC6 ultramicrotome. The sections were stained with 0.5 % toluidine blue, mounted in DPX and observed with a Leica DMR light microscope. For the morphometric analysis, eighteen different sections from each treatment (3 plants per treatment) were studied. The thickness occupied by palisade parenchyma (PP), spongy parenchyma (SP), adaxial epidermis (AdE), abaxial epidermis (AbE), mesophyll thickening (MT) and stomatal opening in the leaves, was measured and expressed in microns using ImageJ software.

Picture 3. Program logo (left) and *Cistus albidus* leaf section image (right).

2.9. Statistical analysis

In the experiment, 50 plants were randomly attributed to each treatment. The data were analysed by one-way ANOVA using the SPSS 20.0 software (SPSS Inc., 2002). Treatment means were separated with Duncan’s Multiple Range test (P≤0.05).

3. Results

3.1. Growth

In well-irrigated plants growing in compost substrate (CpWW), shoot biomass accumulation (shoot DW) was lower than in their counterparts grown in commercial substrate (CWW) (Figure 1a). In water stressed plants in the commercial substrate shoot DW values were significantly lower than in well-irrigated plants, while no significant differences were observed between well-irrigated and water-stressed plants in compost. Root dry weight values were similar in all treatments (Figure 1b). Plant height was less affected due to the substrate than shoot DW during the stress period, but height decreased as a result of water stress in the commercial substrate (Figure 1c).

At the end of the recovery period, CpWS plants showed the lowest values for shoot DW (Figure 1d), while the plants grown in commercial substrate (CWW and CWS) had the highest values. In the case of root DW, the greatest differences were found between CWW and CpWS plants (Figure 1e). At this time, the highest plants were those from CWW and the lowest from CpWS (Figure 1f), while the respective heights of CWS and CpWW were similar. In general, all the plants were higher after recovery than during the water stress period.

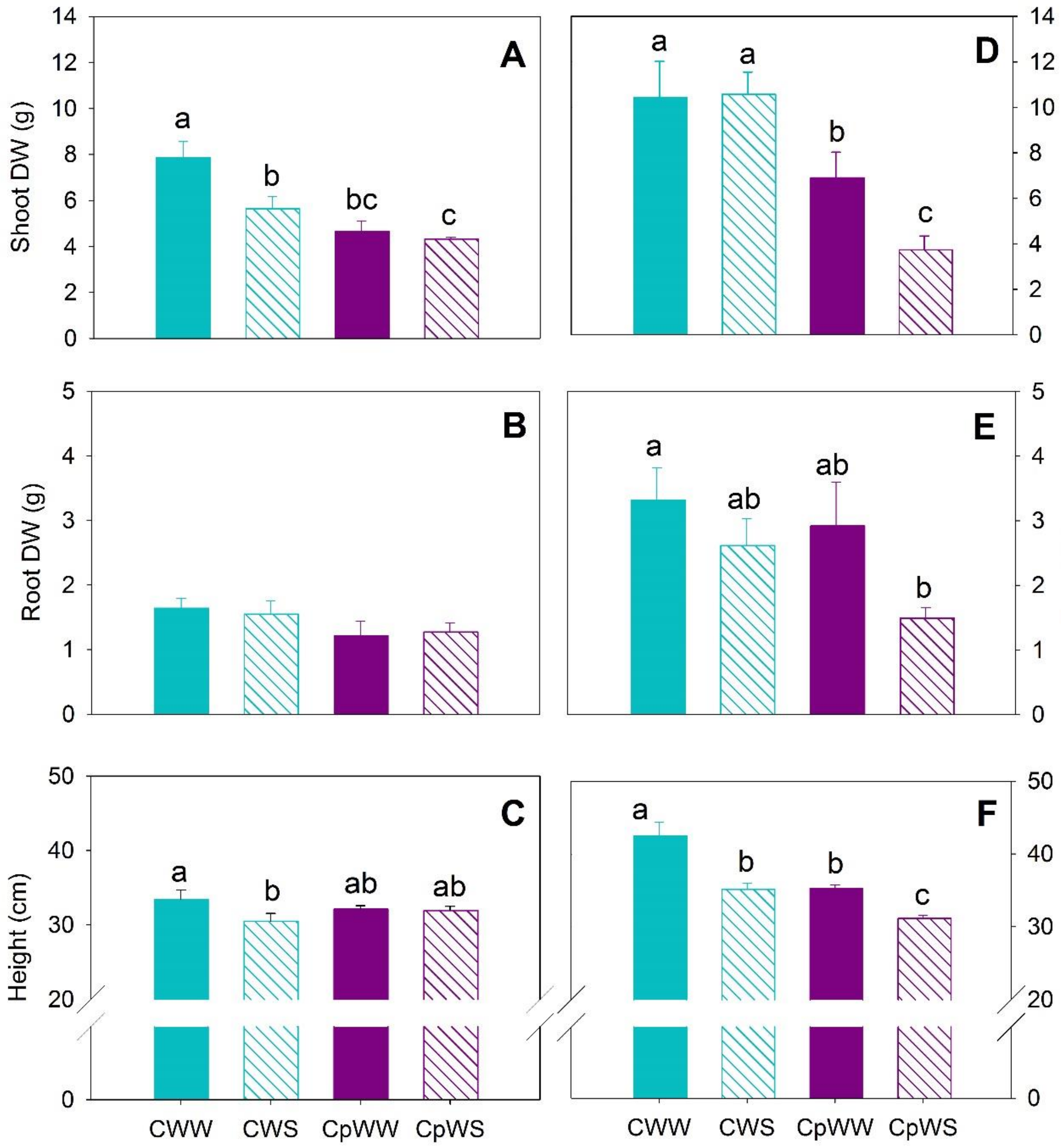


Figure 1. Shoot dry weight, root dry weight and height of Cistus albidus plants grown on commercial (C) and compost (Cp) substrates and subjected to well-watered (WW) and water stressed (WS) at the end of water-stress period) (A, B, C) and at the end of water recovery period (D, E, F). Values are means of five plants. Different letters indicate significant differences between treatments according to the Duncan0.05 test. The vertical bars indicate standard errors.

3.2. Mineral content

The minerals Al, B, Ca, Cu, Li, Mg, Mn, Na, P, Rb, K, S, Sr and Zn were found in higher proportions in the compost substrate than in the commercial substrate, especially in the case of Al, Mn, Cu and Zn. By contrast, the Cr, Fe and Ni contents were lower in the compost substrate (Table 1).

Table 1. Mineral content of the commercial (C) and compost (Cp) substrates at the beginning of the experiment. Different letters indicate significant differences between treatments according to the Duncan$_{0.05}$ test.

	Treatments	
	C	***Cp***
Al (mg/Kg)	1005.38 b	1841.07 a
B (mg/Kg)	11.41 b	42.46 a
Ca (g/100g)	1.14 b	3.70 a
Cr (mg/Kg)	25.51 a	5.35 b
Cu (mg/Kg)	7.95 b	204.23 a
Fe (mg/Kg)	1904.26 a	632.82 b
K (g/100g)	0.25 b	1.02 a
Li (mg/Kg)	1.31 b	4.98 a
Mg (g/100g)	0.30 b	0.76 a
Mn (mg/Kg)	53.94 b	418.24 a
Na (g/100g)	0.14 b	0.30 a
Ni (mg/Kg)	6.85 a	5.15 b
P (g/100g)	0.04 b	1.74 a
Rb (mg/Kg)	4.82 b	10.66 a
S (g/100g)	0.30 b	0.66 a
Sr (mg/Kg)	28.66 b	164.33 a
Zn (mg/Kg)	13.53 b	1751.38 a

In the leaves of plants grown in compost substrate, the levels of Ca were lower and those of K, Na and S higher than in the leaves of plants grown in commercial substrate. When water stress was applied, the Ca, Cu, Mg and Rb contents were lower in the commercial substrate, while only the Zn content decreased in the plants grown in compost substrate (Table 2a). At the end of experiment, after the water stressed plants had been re-watered, the levels of Ca, Fe, P and Sr were lower in the plants from the compost treatments while K, Na, Rb, S and Zn content increased in the same plants, especially K and Na in CpWS (Table 2b).

Table 2. Leaf mineral content of Cistus albidus plants grown on commercial (C) and compost (Cp) substrates and subjected to well-watered (WW) and water stressed (WS) at the end of the water stress period (A) and at the end of the recovery period (B). Values are mean ± SEM (n = 5 plants). Means within a row without a common lowercase letter are significantly different by $Duncan_{0.05}$ test.

a	CWW	CWS	CpWW	CpWS
Al (mg/L)	60.05±7.00	29.50±8.00	77.50±21.50	22.00±1.00
B (mg/L)	165.35±3.50 ab	158.50±1.50 b	153.00±11.50 b	178.00±3.00 a
Ca (mg/L)	8551.50±869.50 a	6031.50±290.50 b	3213.00±576.00 c	2953.50±317.00 c
Cu (mg/L)	12.95±1.50 a	5.00±1.00 b	10.00±3.50 ab	6.00±1.50 ab
Fe (mg/L)	68.50±7.50	54.50±5.00	133.00±88.00	60.00±12.50
K (mg/L)	31883.00±2281.00 b	27151.50±504.50 b	43061.50±1738.00 a	47836.50±880.00 a
Mg (mg/L)	3542.50±238.00 a	2546.00±231.00 b	3551.00±426.00 a	3628.00±95.50 a
Mn (mg/L)	117.00±3.50	123.50±28.50	70.50±4.50	104.50±8.50
Na (mg/L)	1385.50±195.50 bc	749.00±33.50 c	2015.00±422.50 a	2873.50±331.50 a
P (mg/L)	6705.50±637.50	5865.00±341.50	5100.50±834.50	4760.00±351.00
Rb (mg/L)	25.50±1.50 a	19.60±0.50 b	27.00±1.00 a	30.50±0.50 a
S (mg/L)	1654.50±257.00 b	1592.50±206.50 b	2556.00±92.00 a	3107.50±269.50 a
Sr (mg/L)	44.00±4.00	30.50±6.00	31.50±7.50	26.00±3.00
Zn (mg/L)	88.00±13.50 ab	59.50±9.00 b	118.50±26.50 a	97.50±6.50 b

b	CWW	CWS	CpWW	CpWS
Al (mg/L)	20.50±7.50	22.50±4.50	34.50±7.00	30.00±5.00
B (mg/L)	180.50±6.00	175.00±4.00	185.00±15.50	164.50±9.00
Ca (mg/L)	11264.00±1004.00 a	11137.50±739.50 a	3381.50±273.00 b	3169.00±409.50 b
Cu (mg/L)	3.00±0.50	4.00±0.00	5.00±1.00	4.00±0.50
Fe (mg/L)	53.50±4.00 ab	69.00±4.00 a	43.00±11.00 bc	32.00±2.00 c
K (mg/L)	22652.50±1531.50 d	31999.50±975.50 c	50894.00±2653.50 b	65004.00±5578.00 a
Mg (mg/L)	2792.00±147.50	3459.50±154.00	3383.50±120.50	3804.00±451.50
Mn (mg/L)	72.00±7.00	213.50±20.50	80.00±13.00	103.00±10.50
Na (mg/L)	626.00±110.50 c	1077.00±133.50 c	1784.50±330.00 b	4079.00±596.00 a
P (mg/L)	4746.50±333.50 b	6953.00±370.00 a	5613.00±608.50 ab	5246.50±801.00 b
Rb (mg/L)	17.00±1.00 c	22.50±1.00 c	31.00±1.50 b	39.25±3.50 a
S (mg/L)	1926.50±66.50 b	2753.00±171.00 b	4358.50±605.00 a	5026.00±504.00 a
Sr (mg/L)	21.50±3.50 a	28.00±2.00 a	7.50±2.00 b	11.50±2.50 b
Zn (mg/L)	71.50±5.50 b	86.00±4.00 b	177.50±36.00 a	138.00±30.00 b

3.3. *Water stress development and recovery. Survival*

When irrigation was stopped, the time taken to reach a leaf water potential of around -3.0 MPa (threshold value at which the water stress was established) was different between the plants grown in commercial substrate and those grown in compost substrate (Figure 2). In the case of commercial substrate, the response of plants to the absence of irrigation was faster (13 days compared with the 53 days taken by compost-grown plants to reach a similar level of water stress) (Figure 2), probably because the compost substrate had a greater water retention capacity. Once all plants were re-watered, the recovery period needed to reach a leaf water potential value of around -0.9 MPa (well-watered plants) depended on the substrate used: 13 d in the case of the commercial substrate (C) and 20 d for the compost (Cp) (Figure 2).

At the end of the experiment, plant survival in the different treatments was: <66% (CpWW) <69% (CpWS) and 100% in the rest of the plants (CWW, CWS).

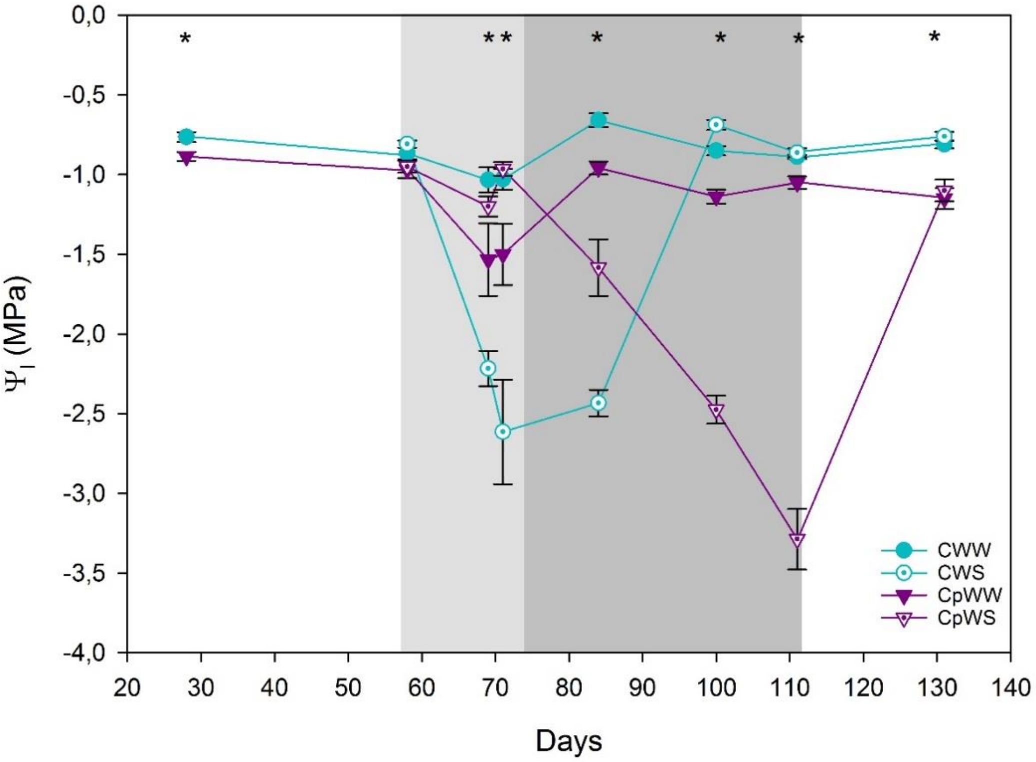


Figure 2. Leaf water potential (Ψl) of Cistus albidus plants, grown on commercial (C) and compost (Cp) substrates and subjected to well-watered (WW) and water stressed (WS) throughout the experiment period. Asterisks indicate statistically significant differences between treatments by $Duncan_{0.05}$ test. The light gray stripe indicates the water stress period of the plants growing in commercial substrate and the dark gray stripe indicates the period in which their recovery begins. Water stress period of the plants growing in compost is the sum of both stripes. The vertical bars indicate standard errors.

3.4. Osmotic adjustment

As regards osmotic potential at full turgor (Ψ_{100s}), this was significantly lower in plants grown in compost substrate (Cp) (Figures 3a,b), an effect that became more pronounced with water stress (CpWS). No osmotic adjustment occurred in the plants grown the commercial substrate and submitted to water stress (Figure 3a). The recovery of Ψ_{100s} in plants grown in compost respect to stress period was significant, especially in the plants that had been exposed to water stress, in which the parameter reached values similar to that of well-watered plants (Figure 3b).

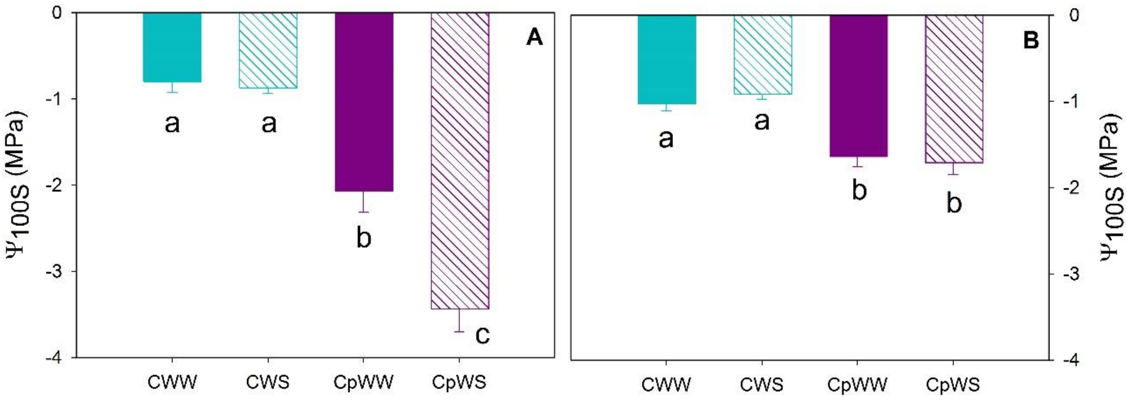


Figure 3. Osmotic water potential at full turgor (Ψ_{100s}) of Cistus albidus plants, grown on commercial (C) and compost (Cp) substrates and subjected to well-watered (WW) and water stressed (WS), at the end of water-stress period (A), and at the end of water recovery period (B). Values are means of five leaves. Different letters indicate significant differences between treatments according to the Duncan$_{0.05}$ test. The vertical bars indicate standard errors.

3.5. Gas exchange and leaf temperature

The stomatal conductance (g_s) values of well-watered plants (Figure 4a) grown in commercial substrate was significantly higher than in those grown in compost under the same irrigation conditions, with mean values throughout the experiment of 150 mmol $m^{-2}s^{-1}$ and 50 mmol $m^{-2}s^{-1}$, respectively (Figure 4a). When water stress was induced, the stomatal conductance fell drastically in the plants of the commercial substrate. However, the difference in g_s between well-watered plants and water-stressed plants was less pronounced for plants grown in compost, showing lower values in both treatments (Fig. 4a). There was no recovery of g_s values in CWS, while in plants grown in compost substrate the values in CpWW and CpWS were similar (Figure 4a).

The behaviour of P_n was similar to that of g_s (Figure 4b), although the differences between CpWW and CpWS were more pronounced than those found for g_s (Figure 4b).

Water stress induced an increase in leaf temperatures with respect to well-watered plants, especially in the commercial substrate (Figure 4c). During recovery period, leaf T values slightly decreased in all treatments with respect to the values observed during the water stress period, and no differences between treatments were observed (Figure 4d).

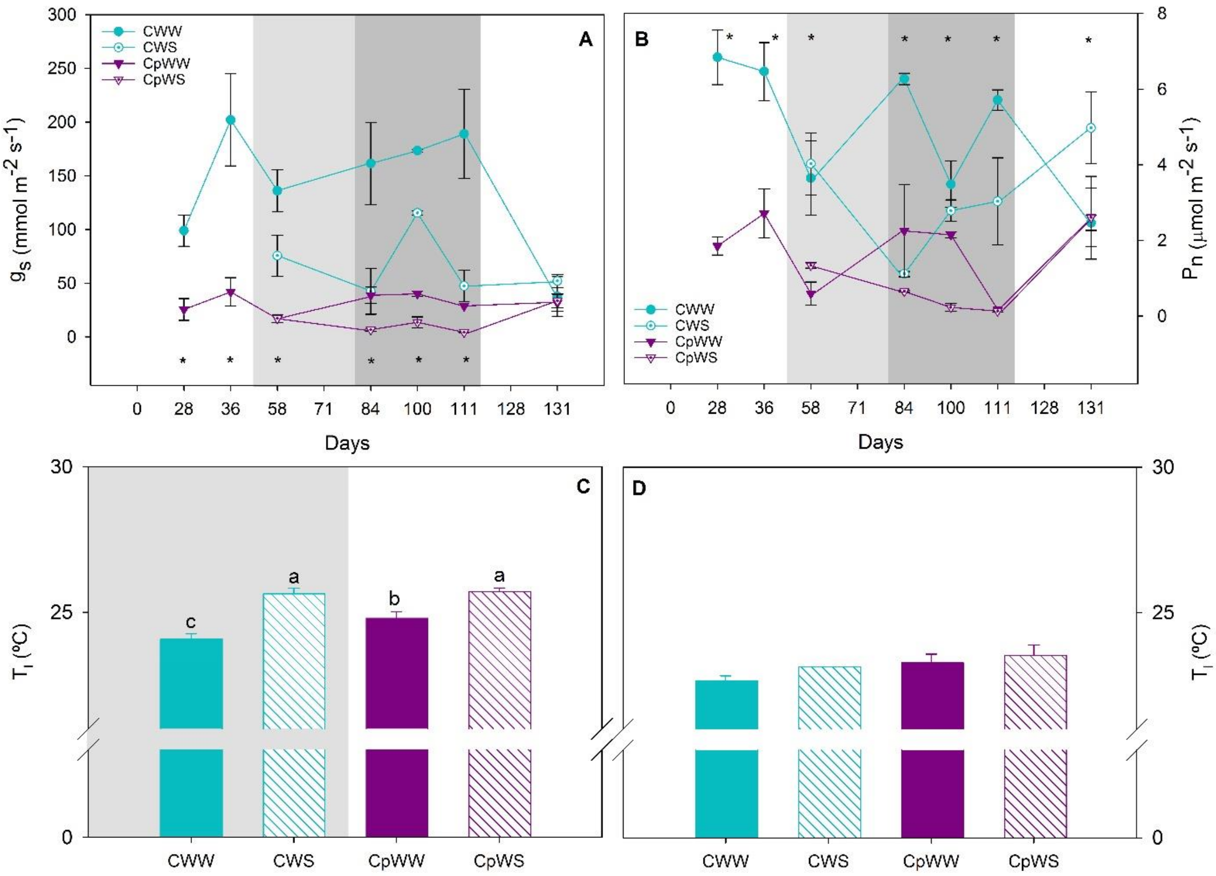


Figure 4. Stomatal conductance (gs, A), net photosynthetic rate (Pn, B) and leaf temperature (T_l, C and D) of Cistus albidus plants, grown on commercial (C) and compost (Cp) substrates, subjected to well-watered (WW) and water stressed (WS) throughout the experiment period (water stress period and recovery period). T_l is represented at the end of the stress period (C) and at the end of the recovery period (D). Asterisks in Fig. 4A and B indicate statistically significant differences between treatments by $Duncan_{0.05}$ test. The light gray stripe indicates the water stress period of the plants growing in commercial substrate and the dark gray stripe indicates the period in which their recovery begins. Water stress period of the plants growing in compost is the sum of both stripes. The vertical bars indicate standard errors. Different letters in Fig. 4C and D indicate significant differences between treatments according to the $Duncan_{0.05}$ test.

3.6. Chlorophyll fluorescence and lipid peroxidation

In well-watered plants there were no significant differences in lipid peroxidation values caused by the substrate used (Figure 5a). When water stress was imposed, lipid peroxidation values increased in plants grown in both substrates. After the recovery period, no statistically significant differences were observed between treatments (Figure 5b). In plants grown in compost, there is no decrease in these values in relation to the water stress period.

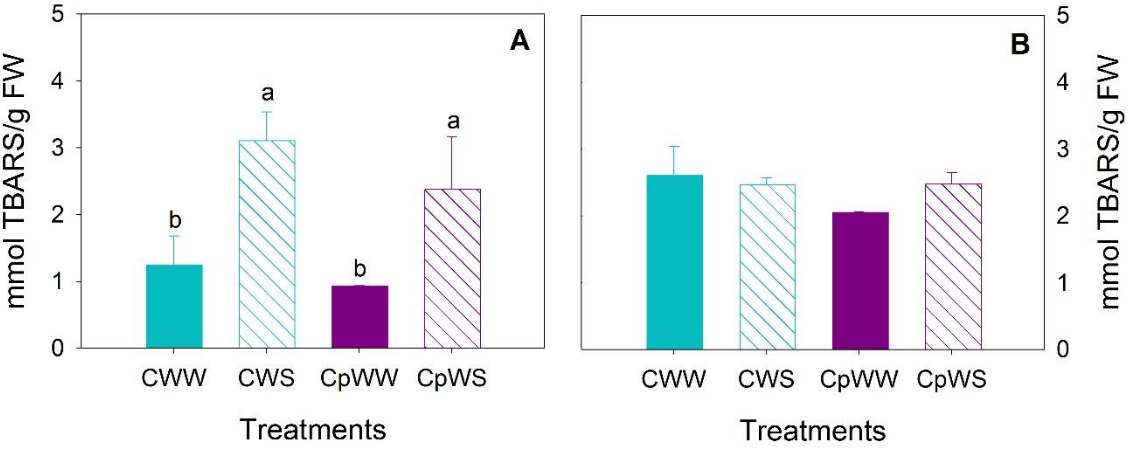


Figure 5. Lipid peroxidation of Cistus albidus plants, grown on commercial (C) and compost (Cp) substrates and subjected to well-watered (WW) and water stressed (WS), at the end of water-stress period (A) and at the end of water recovery period (B). Values are means of three plants. Different letters indicate significant differences between treatments according to the Duncan$_{0.05}$ test. The vertical bars indicate standard errors.

Chlorophyll fluorescence analysis to know photochemical efficiency of plants at the end of both periods is shown in table 3. The maximum quantum yield of PSII (Fv/Fm) were around 0.80 for the control plants grown in both substrates, while in their water-stressed counterparts, Fv/Fm decreased in commercial substrate (0.62) and, to a lesser extent (0.78), in compost (Table 3) The effective quantum yield (Y(II)) values were higher in well-watered plants grown in compost substrate (Table 3). Water stress induced a significant decrease in Y (II) in plants grown in both substrates, especially in commercial substrate. With regard the non-photochemical quenching (qN), this decreased in the CWS plants compared to the rest of the treatments (Table 3). When the plants were re-watered, no statistical differences in Fv/Fm between CWW and CWS treatments were observed (Table

3), however, the CpWW plants had a lower Fv/Fm than the CpWS plants. In this time, the Y (II), qP and qN values of the plants that had been under water stress in both substrates reached similar values to those of well irrigated plants (Table 3).

Table 3. Leaf chlorophyll fluorescence (Fv/Fm, efficiency of PSII; Y(II), quantum yield; qP, photochemical quenching; and qN, non-photochemical quenching) of Cistus albidus plants grown on commercial (C) and compost (Cp) substrates and subjected to well-watered (WW) and water stressed (WS) at the end of the water stress period (A) and at the end of the recovery period (B). Values are means ± SEM (n=3 plants). Means within a row without a common lowercase letter are significantly different by $Duncan_{0.05}$ test.

	a	**CWW**	**CWS**	**CpWW**	**CpWS**
Water-stress period	**Fv/Fm**	0.805±0.008 a	0.721±0.026 c	0.814±0.005 a	0.785±0.009 b
	Y(II)	0.264±0.024 b	0.076±0.003 c	0.393±0.033 a	0.293±0.030 b
	qP	0.376±0.028 b	0.105±0.042 c	0.540±0.046 a	0.421±0.040 b
	qN	0.522±0.006 a	0.156±0.013 b	0.454±0.060 a	0.459±0.014 a
	b	**CWW**	**CWS**	**CpWW**	**CpWS**
Recovery-period	**Fv/Fm**	0.805±0.003 a	0.781±0.005 a	0.633±0.059 b	0.741±0.001 a
	Y(II)	0.407±0.029 a	0.386±0.040 a	0.229±0.030 b	0.300±0.010 b
	qP	0.562±0.033 a	0.572±0.053 a	0.441±0.004 b	0.518±0.022 ab
	qN	0.439±0.048 b	0.512±0.040 b	0.542±0.054 ab	0.649±0.013 a

3.7. Leaf anatomical changes

Water stress and substrate induced some changes in the leaf anatomy of *C. albidus* plants at the end of water stress period (Table 4). Compost-grown plants underwater stress conditions (CpWS) presented an increase in the percentage of palisade parenchyma, adaxial epidermis and mesophyll thickening than well-irrigated (CpWW). In the case of plants grown in commercial substrate, the proportion of the different tissues by water stress (CWS) decreased comparing with CWW (table 4). At the end of the stress phase, we verified how the leaves of the plants found in the well-watered treatments were flatter compared to the leaves of the plants of the treatments in water stress, which were sometimes bent, forming crypts in the abaxial zone. In both compost treatments and in the commercial stressed water substrate, the stomata clearly closed; unlike those of the plants grown in commercial substrate not subjected to water stress (Table 4).

Table 4. The percentage of area occupied by palisade parenchyma (PP), spongy parenchyma (SP), adaxial epidermis (AdE), abaxial epidermis (AbE), mesophyll thickening (MT) and stomatal opening in leaves of Cistus albidus plants grown on commercial (C) and compost (Cp) substrates subjected to well-watered (WW) and water stressed (WS) at the end of water stress period. Values are means ± SEM (n=3 plants). Means within a row without a common lowercase letter are significantly different by Duncan$_{0.05}$ test.

	CWW	**CWS**	**CpWW**	**CpWS**
AdE	26.337 ± 1504 a	17.715 ± 4064 b	16.086 ± 0.783 b	24.712 ± 0.988 a
PP	69.279 ± 1775 b	64.150 ± 14.717 c	68.644 ± 1198 b	93.547 ± 2000 a
SP	71.240 ± 3768 a	27.502 ± 6309 b	77.262 ± 1323 a	72.644 ± 1348 a
AbE	11.761 ± 0.549 b	7189 ± 1649 c	23.132 ± 1397 a	10.546 ± 0.338 b
MT	181.952 ± 3725 b	142.868 ± 32.776 c	181.905 ± 2675 b	204.322 ± 1978 a
Stomata	25.996 ± 0.361 a	9626 ± 2208 b	7412 ± 0.401 c	5558 ± 0.223 d

4. Discussion

The compost substrate used was characterized by its higher heavy metal content (Al, Cu, Mn and Zn) and lower level of Fe compared with the commercial substrate (Table 1). These characteristics conditioned the behavior of the plants both under good irrigation and water stress conditions. Metal toxicity is known to have multiple direct and indirect effects on plants that affect all physiological functions (Rucinska-Sobkowiak, 2016). These toxic effects depend on the external concentration, exposure time and genotype of the plant. Some plants prevent heavy metals from entering the roots (Ghosh and Singh, 2005), while others confine and detoxify them in a controlled manner (hyperaccumulators) (Rascio and Navari-Izzo, 2011). The excluder group includes the majority of plant species, like Cistus, that limit the translocation of heavy metals and maintain low levels of contaminants in their aerial tissues over an extensive range of soil concentrations (Millaleo et al., 2010). As can be seen, the higher levels of Al, Cu, Mn and Zn in the substrate (Table 1) did not translate into higher levels of these minerals in the leaves of the plants grown in compost (Table 2).

Nevertheless, changes in biomass parameters are indicators of plant tolerance to the presence of heavy metals (Ingrouille and Smimoff, 1986). In our experiment, shoot DW was noticeably lower in plants grown in compost compared with those grown in commercial substrate, even when well-irrigated. Such lower growth may be related with an excess of metal ions in the medium, factors that regulate the entry and exit of water in plants, which affect the cell walls and consequently growth and expansion (Barceló and Posschenrieder, 1990; Rucinska-Sobkowiak, 2016).

In the case of plants grown in commercial substrate, the decrease in biomass was directly due to the imposed water stress, which would cause a water deficit in these plants, determining cell expansion and photosynthesis related to biomass growth (Sánchez - Blanco et al., 2002; Sánchez-Blanco et al., 2019).

It is known that stomatal closure is one of the fastest responses to water stress in plants (Anjum et al., 2011) and that it may reduce photosynthesis due to the lack of CO_2 fixation. In compost-grown plants, the stomatal closure occurred both in plants subjected to water stress and in well irrigated plants (Figure 4 and Table 4), probably due to the excess of minerals in the substrate, as has been observed in several studies that have described significantly reduced stomatal conductance caused by the effect of minerals (Kasim, 2006; Sagardoy et al., 2010). Stomatal closure prevents transpiration, resulting in an increase in leaf temperature (Leinonen et al., 2006; Gómez-Bellot et al., 2015), even in well-watered plants under compost substrate.

As we have previously pointed out, stomatal closure is a mechanism used by plants to reduce water loss in the face of water stress (Bañón et al., 2011; Gómez-Bellot et al. 2013a,b; Sánchez-Blanco et al., 2002; Sánchez-Blanco et al., 2019). In our experimental conditions, furthermore, compost added an osmotic component that induced a stronger response from the plants. Reductions in stomatal conductance have previously been observed in metal-exposed plants (Disante et al. 2011). All of these physiological responses may result in restriction of water uptake in metal-stressed plant, which aggravate the consequences of drought stress (Perfus-Barbeoch et al., 2002). Furthermore, in addition to increasing by stomatal closure, leaf temperature can also be increased by the culture medium (Maes and Steppe, 2012).

As has been previously mentioned, stomatal closure is a dominant factor that limits gas exchange during water deficit (Davies and Gowing, 1999). A decrease in stomatal conductance normally correlates with a decrease in leaf water potential during the period of water stress (Gomes et al., 2004; Álvarez et al., 2018), but a stomatal closure may also occur without large changes in leaf potential. In fact, once the stomata are closed, leaf water potential values are maintained (Schultz, 2003). Both types of behavior were observed in our treatments, the first in the plants grown in commercial substrate and submitted to water stress and the second during the first two weeks of the experiment in the plants grown in compost (Figure 4). As a result of stomata reduction, net photosynthesis was reduced due to decreased CO_2 availability at the chloroplast level (Chaves et al., 2009), as seen in many other ornamental species submitted to water deficit (Sánchez-Blanco et al., 2019; Álvarez et al., 2013).

Leaf water potential and osmotic potential are good indicators of water stress. A decrease in osmotic potential at full turgor indicates whether or not osmotic adjustment has occurred due to water stress (Álvarez and Sánchez-Blanco, 2015). The compost-grown plants showed osmotic adjustment even without water stress, unlike the plants grown in commercial substrate and subjected to water stress, which showed no osmotic adjustment. Perhaps, the water stress developed so rapidly (2 weeks) that osmotic adjustment did not occur even though the leaf water potential reached values of around -3.0 MPa. In this sense, Alegre (2000) demonstrated that the degree of osmotic adjustment depends on the species, and speed of the imposed stress. On the other hand, the decrease of Ψ_{100s} in compost-grown plants when water stressed may have been the result of an accumulation of solutes due to the mineral content of the substrate (Acosta-Motos et al., 2017). Changes in K followed by Na in tissue plant may contribute substantially to osmo-regulation (Ortuño et al., 2018), which would have been the case in the plants grown in the compost substrate (Table 2).

After restoring adequate irrigation to the water-stressed plants, the recovery of several parameters was studied. According to Dodd et al. (2015), re-watering greatly affects plant physiology, altering the export of xylem-borne chemical signals from the root to the shoot (Gómez-Cadenas et al., 1996; Hansen and Dorffling, 2003). Thus, the study of stress/recovery responses is instrumental for achieving a better understanding of the mechanisms to adapt different environments (Sapeta et al., 2013).

In our case, the leaf water potential of the previously stressed plants reached values close to those of the plants that had been well watered throughout the experiment, although the time taken differed with the type of substrate (Figure 2). However, the same did not occur in the case of stomatal conductance and photosynthesis. According to Chaves et al. (2009), after a period of water stress, the recovery of photosynthesis may depend on the degree and speed of the decrease in photosynthesis, which may be related to the substrate, as we have already mentioned, although such behavior has been poorly studied.

In general, plants subjected to mild stress recover quickly, but plants subjected to severe water stress the recovery is progressive, slow, and sometimes incomplete, 40-60% of the maximum rate of photosynthesis obtained under control conditions (Souza et al., 2004; Flexas et al., 2006; Miyashitaet al., 2005). In these cases, it has been suggested that sustained photoprotection and /or oxidative stress may be possible causes of this incomplete recovery (Gallé et al., 2007).

Turning our attention to the morphological changes that occurred as a consequence of both substrate and water stress (table 4), it can be observed that there was an increase in the thickness of the parenchyma, adaxial epidermis, and mesophyll in water-stressed plants grown in compost substrate. The higher concentrations of minerals in the leaf (K, Na, S and Zn) along with the reduction in water availability increased thickness of leaf, leaf tissues and modify the location of stomata in crypts. These structural changes are part of the adaptive mechanisms to abiotic stresses, such as water stress, to minimize water loss by transpiration (Acosta- Motos et al., 2017; Guha et al., 2010). However, the opposite occurred in the leaves of commercial-grown plants, where a decrease in the proportions of the different tissues evaluated with the reduction in soil moisture was observed. This decrease can be possibly due to the decrease in cell size in response to water stress (Zhang et al., 2012), which is commonly associated with a lower water stress tolerance (Ristic & Cass, 1991).

On the other hand, although the compost increased the leaf Na content, it was not accompanied by a decrease in K accumulation; quite the contrary, an increase was recorded under stress conditions, would indicate that the compost was not especially harmful (Ortuño et al., 2018). During the recovery period, the differences in the mineral content between the stressed plants and those that were well-watered throughout the experiment were more evident in compost substrate (higher Na, K, Rb and S levels and lower Fe and Ca levels) than in commercial plants.

The chlorophyll fluorescence is an important method of evaluating plant physiological status during the cultivation cycle (Toivonen and Vidaver, 1988; Murchie and Lawson, 2013). The Fv/Fm value may reflect the potential efficiency of PSII. The values of Fv/Fm were significantly lower under water stress, especially in the plants grown in commercial substrates, which could be due to photodamaged in PSII, diminishing the maximum photosynthetic capacity of plants (Porcar-Castell et al., 2014). Both photochemical values of fluorescence Y(II) and qp have been used as indicators of the effect of abiotic stresses in the photosynthetic apparatus (Cantabella et al., 2017). In our experiment, qP and Y(II) in leaves decreased under water stress in both substrates, indicating that the photosynthetic electron transport activity and the energy used in photosynthesis decreases under these conditions (Fryer et al., 1998), however, the qN value did not increase with water stress. This is against expectations, since stressed plants dissipate radiant energy as sensible heat (increasing qN) to protect leaves from photooxidative damage, acting as a self-protection mechanism in leaves (Herrera, 2000; Maxwell and Johnson, 2000). Then, we can hypothesize that the capacity of dissipating light energy by non-photochemical means of CWS plants was damaged strongly due to water stress, suggesting that these plants had photosynthetic disorders at the photochemical level.

In terms of qP and Y(II), the plants grown in compost substrate responded slightly better than those grown in the commercial substrate and qN was no affected by water stress, supporting the idea that water stress did not harm the photosynthetic system of CpWS plants. This was probably due to a process of acclimatization, as reflected in the photochemical quenching parameters. It is possible that the response to the presence of metals actually make plants less susceptible to drought (de Silva et al., 2012). On the other hand, the higher lipid peroxidation values found in stressed plants possibly reflect greater damage to membranes.

The recovery period was a decisive moment for the survival of water-stressed plants, although the recovery of optimal conditions may be perceived as a new stress, as observed in other studies in plants exposed to saline stress (Hernández and Almansa, 2002, Acosta-Motos et al., 2015). In our experiment, under commercial substrate, in the plants that did not survive the rehydration was perceived such a stress that exceeded the capacity of recovering of these photochemical mechanisms, resulting in the death of the plants. On the contrary, the surviving plants recorded good values of Fv/Fm, Y(II) and qP recovered their capacity of protecting against photooxidative damage by dissipating heat, since CWW and CWS presented similar qN values. In the case of compost substrate, the fluorescence values were again optimal in stressed plants, indicating that the CpWS plants were photochemically healthy, and the drop in Pn was a result of the reduction in gs (Table 3).

In conclusion, high levels of heavy elements (Cu, Zn, Al, Mn, etc.) in an unconventional substrate, such as compost, negatively affected plant growth, even when the plants were well irrigated. Severe water stress (-3.0 MPa) was slower to develop in compost plants due to limitations in their transpiration process. Different soil substrates under water stress affected leaf anatomy differently, whereas plants grown in commercial substrate showed a decrease in thickness of the leaf tissue, in compost, an increase in the proportion of adaxial epidermal and parenchymal tissues occurred.

The relationship between stomatal conductance and water status depended on the substrate, which also determined the time taken for the plants to recover. In both substrates used, the water potential recovered before the photosynthetic activity. The restoration of irrigation was perceived as a new stress, especially in plants grown in commercial substrate, as reflected in the photochemical mechanisms.

Chapter 1.2

Mycorrhizal inoculation on compost substrate affects nutritional balance, water uptake and photosynthetic efficiency in *Cistus albidus* plants submitted to water stress

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Abstract

The use of composted manure as alternative substrate can be suitable to produce ornamental potted plants. However, under water stress this substrate can result an additional stress for plants due to its physico-chemical properties. In these conditions, mycorrhizae application can improve water and nutrients efficiency. The experiment was carried out in a growth chamber with a first phase (I) in which both inoculated and non-inoculated *Cistus albidus* plants at two substrates (commercial and mixtures of composted manure) were well-irrigated and a second phase (II) in which the plants were submitted to water stress. *Glomus iranicum* var. *tenuihypharum* was well established in cistus roots, but water stress hindered mycorrhizal proliferation in compost, which resulted in plants with smaller leaf and root biomass. The plants in compost had the highest Cl, K, Na, P and Zn contents in leaf, mycorrhizae reduced the Na and increased phosphorus, especially when the substrate was well watered. Water stress decreased leaf water potential (Ψ_l), and mycorrhizae induced higher Ψ_l values in both substrates. Compost induced leaf osmotic adjustment, lower gas exchange and photochemical quenching parameters (F_v/F_m, Y (II)) values. Mycorrhizal plants had higher Y (II) and qP values than non-inoculated plants. Compost decreased relative chlorophyll content in both phases, but in inoculated plants these values increased under water stress. *C. albidus* plants growing in compost maintain a good nutritional balance and efficient osmotic regulation. Under water stress, plants suffer more stress than plants in commercial substrate, as reflected by the lipid peroxidation and Pn values.

Keywords: AMF; Compost substrate; Heavy metals; Nutrients; Osmotic adjustment; Photosynthetic activity.

1. Introduction

Materials such as peat and natural soils have frequently been used as substrates in nurseries for ornamental plant production (Pagés and Matallana 1984). However, the depletion of non-renewable resources such as peat together with its high price in the market has spurred the use of alternative materials as growth substrates (Abad et al. 2001). The search for alternative substrates to produce ornamental plants has led us to use diverse native materials, which can be environmental friendly and have optimal physicochemical properties for the plant. Traditionally, residues such as urban solid wastes and even green wastes were considered as non-desirable substrates. Some studies, however have shown that, after composting, these organic residues, can be used as growth media instead of peat with very good results (Piamonti et al. 1997; García-Gómez et al. 2002). Nevertheless, while slight variations in particle size, texture and mineral composition are less important when the material is used in landscaping, these factors combined with other components may be of significant importance in a limited volume as plant container, since they can affects the growth rate, nutrition and quality of the plant (Larcher and Scariot 2009).

Although a wide range of wastes have been studied as peat substitutes, there is not enough information concerning the use of pruning waste compost as an alternative to commercial substrates in ornamental plant production (Benito et al. 2005; Hernandez-Apaolaza et al. 2005). It may contain certain phytotoxic ions and heavy metals. In such cases, this type of substrate could be used for landscaping and revegetation projects using ornamental plants where the impact is not as important as it would be in other crops for human consumption. All of this requires a deeper study to know physical and chemical characteristics to provide a reservoir for water and nutrients (Larcher and Scariot 2009).

Cistus albidus L. is a typical shrub of the Mediterranean climate. In landscaping, the grayish-green tonality of its leaves is very useful for making compositions contrasting different types of greens. These plants began to be widely used in gardening and landscaping due to their rusticity and attractive blooms. Cistus species have evolved mechanisms to resist drought stress and to regenerate after fire, ecological characteristics that affect the evolution of Mediterranean vegetation in general (Saura-Mas and Lloret 2007). Concretely, *C. albidus* responds to water deficit by developing avoidance mechanisms based on stomatal closure, a reduction in leaf area and epinasty, which can be considered as complementary mechanisms for regulating transpiration (Sánchez-Blanco et al. 2002). All of these responses have been recognized as important mechanisms for confronting drought. Cistus species may form both ectomycorrhizae and arbuscular mycorrhizae (Smith and Read 2008), although most plant genera form only one type of association. The dual mycorrhizal status is also present in other species such as *Populus* sp., *Salix* sp., *Eucalyptus* sp. (Smith and Read 2008).

Picture 1. *Cistus albidus* L. plant

Arbuscular mycorrhizal fungi (AMF) enhance growth under normal and stress conditions by affecting physiological and nutritional processes. Numerous authors have reported, for example, that AMF are able to increase the absorption of water and nutrients (Aroca et al. 2013; Ahanger et al. 2014; Gómez-Bellot et al. 2015a). Not all AMF behave identically in a given environment, however, nor is a given fungus the most appropriate for all environments. It is therefore important to know the biological responses of the different fungi in order to use the most appropriate one, depending on the species, cultivar, type of stress and conditions (Mardukhi et al. 2011; Navarro et al. 2011). *Glomus* iranicum Btaszk.*,* Lovács & Balázs var*. tenuihypharum* sp. (mycorrhizal inoculant Mycogrowth, SYMBORG S.L.) is an arbuscular fungus that has been used in other Mediterranean species under different abiotic stress conditions, which was effective in terms of improving growth and esthetic value, stimulation of plant physiological parameters such as stem water potential and stomatal conductance, etc Gómez-Bellot et al. 2015a,b).

The objective of this work was to evaluate the use of compost manure as a substrate for producing *C. albidus* plants in pots, and to determine whether the fungus *G. iranicum* var*. tenuihypharum*, which is not frequently used in this genus, could provide benefits, especially when the plants are submitted to water stress. The effects of both variables alone (substrate and mycorrhizae) and in combination were evaluated based on parameters that reflect the commercial quality of the plants, such as fresh and dry weight, water status and gas exchange, photosynthetic efficiency and lipid peroxidation.

2. Material and methods

2.1. Plant material and experimental conditions

C. albidus (40) plants, were grown in 14x12x12 cm pots (1.2 l) filled with either Spaghnum peat, coconut fiber and perlite (8:7:1) as a commercial substrate (C), and with a mixture of slurry compost, coconut fiber and perlite (3:6:1) as compost substrate (Cp). Both substrates were improved with Osmocote (14:13:13 N, P, K microelements). 10 plants per substrate were inoculated with *G. iranicum* var*. tenuihypharum* (15 g per 100 mL). The experiment was carried out in a controlled growth chamber, in which the natural temperature changes (23°C/18°C day/night), active light for photosynthesis (350 µmol m^{-2} s^{-1}), photoperiod (16/8 hours light/dark) and relative humidity (60%). Once the *C. albidus* plants were adapted to the chamber conditions, the irrigation treatments consisted in a first phase (phase I) in which both inoculated and non-inoculated plants at two substrates were watered to field capacity (well-irrigated) during eight weeks, and then, a second phase (phase II) in which irrigation was removed to half the plants, causing water stress during four weeks.

Picture 2. Our plants in the controlled growth chamber.

2.2. Fungal colonization

At the end of the experiment, roots were treated to evaluate fungal development. Once cleaned, the roots were immersed in KOH (100°C for 7 min), followed by a bath in H_2O_2 (100°C, 5-6 min) and finally, trypan blue staining (4 min). The percentage of colonization was calculated using the methodology proposed by Kormanik and McGraw (1982). The colored roots were placed on specialized plates for counting and were observed under the magnifying glass by counting 100 fields neatly (positive and negative). The percentage of colonization was calculated using the following formula proposed by Sieverding (1983):

$$\%\, colonization = \frac{number\ of\ colonized\ fields}{total\ number\ of\ fields\ observed} \times 100$$

2.3. Substrate and leaf mineral content

Three substrate samples per treatment were collected at the end of each phase (phase I and phase II). The macronutrient concentrations were determined in a digestion extract with HNO_3:$HClO_4$ (2:1, v/v) by Inductively Coupled Plasma optical emission spectrometer (ICP-OES IRIS INTREPID II XDL). The concentration of Cl was analysed by a chloride analyzer (Chloride Analyser Model 926, Sherwood Scientific Ltd.) in the aqueous extracts obtained by mixing 100 mg of dry sample powder with 40 mL of water before shaking for 30 min and filtering. At the end of experimental period, leaf mineral content was determined in five plants per treatment, as indicated above for mineral soil content.

2.4. Biomass and water relations

To assess biomass, three well irrigated plants at the end of phase I and three non-irrigated plants at the end of phase II were selected and all the substrate was gently washed from their roots. Each plant was then divided separately into leaves, stems and roots and the fresh weight was determined. After drying in an oven at 80°C, until samples reached a constant weight, the dry weights (DW) were obtained.

Picture 3. Scholander chamber.

Seasonal changes in leaf water potential (Ψ_l) and osmotic water potential at full turgor (Ψ_{100s}) during the maximum light hours were determined in four plants per treatment throughout the experimental period. The leaf water potential was determined according to the technique described by Scholander et al. (1965), using a pressure chamber (Soil Moisture Equipment Co., Santa Barbara, CA, USA, model 300), the leaves were pressurized at a rate of 0.03 MPa s^{-1}. The osmotic water potential at full turgor (Ψ_{100s}) was estimated by immersing the petiole in distilled water for 24 hours to achieve complete saturation. After thawing, Ψ_{100s} was measured using a Wescor 5520 vapor pressure osmometer (Wescor Inc., Logan, UT, USA), according to Gucci et al. (1991).

2.5. Gas exchange and thermography

The net photosynthesis rate (P_n) and stomatal conductance (g_s) were determined using a portable gas exchange device, LIICOR LI-6400 (LI-COR Inc., Lincoln, NE, USA). The flow rate of circulating air within the system was approximately 200 mmol s^{-1}, with a sheet vapour pressure deficit to air of about 2 KPa. The CO_2 concentration was fixed at 380 ppm and the photosynthetically active radiation (PAR) at 600 µmol m^{-2} s^{-1}. The measurements were carried out on the same days as the water relations.

At the same time, leaf temperatures were measured using thermography. Thermal images were taken in four plants per treatment with a thermal camera (ThermaCam FLIR-e50 System, Inc., Danderyd, Sweden) which consisted of a 240x180 pixels line scan imager operating in the 7.5e13 mm region, with a noise equivalent temperature difference of 0.05ºC at 30ºC and an accuracy of 2ºC or 2% of the reading. The background temperature, distance of the camera from the canopy, air temperature, emissivity and relative humidity were used as input at the start of each series of measurements; so the camera automatically corrects for atmospheric transmission based on these data. Background temperature was determined as the temperature of a crumpled sheet of aluminium foil in a similar position to the leaves of interest with the emissivity set at 1.0. Emissivity for leaf measurements was set at 0.96 (Leinonen et al. 2006) and the distance at which images were taken was 0.5 m. The images were processed with ThermaCam Reacher Professional 2.10 software (FLIR Quick Report).

2.6. Chlorophyll fluorescence and content.

The relative chlorophyll content was determined periodically in five leaves per treatment with a Minolta SPAD-502 chlorophyll meter (Konica Minolta Sensing Inc., Osaka, Japan), a non-destructive method. Chlorophyll fluorescence measurements were performed with a fluorimeter (IMAGING-PAM M-series, Heinz Walz, Effeltrich, Germany) in three leaves per treatment. After the adaptation of the leaves to the dark (15 min), the maximum and minimum fluorescence values were taken. Kinetic analyses were carried out with actinic light (81 µmol quanta m^{-2} s^{-1} PAR) and repeated pulses of saturating light at 2700 µmol quanta m^{-2} s^{-1} PAR for 0.8 s at 20 s intervals (Clemente-Moreno et al. 2013). Parameters related to both photochemical and non-photochemical processes were measured. With regard to photochemical processes, we measured: quantum yield (Y(II)), photochemical quenching (qP) and efficiency of PSII (Fv/Fm). Non-photochemical processes (NPQ), composed of qN, Y (NPQ) and NPQ/4 components, are related to heat dissipation and are indicators of stress tolerance and have proved to be very sensitive parameters for the early detection of stress conditions by fluorescence imaging (Maxwell and Johnson 2000).

Picture 4. Minolta SPAD-502 chlorophyll meter (Konica Minolta Sensing Inc., Osaka, Japan) (left) and fluorimeter (IMAGING-PAM M-series, Heinz Walz, Effeltrich, Germany) (right).

2.7. Lipid peroxidation

Lipid peroxidation was estimated by determining the concentration of substances that react with thiobarbituric acid (TBARS) following the procedure described in Acosta-Motos et al. (2015). The plant material (0.1 g of leaf) was homogenized in 1 M perchloric acid solution (w/v 1/10). This homogenate was then centrifuged at 14000 g for 10 min, and 0.4 mL of the supernatant was taken and mixed with 1.2 mL of 0.5% TBA. Subsequently, this mixture was incubated at 90°C for 20 min and the reaction was stopped by placing the eppendorf on ice. A final 5 min centrifugation was performed at 10000 g and the absorbance was measured at 532 and 600 nm. The amount of TBARS was calculated from the absorbance value given by the difference between the values obtained at 532 and 600 nm and a molar extinction coefficient of 155 mM^{-1} cm^{-1}.

2.8. Statistical analysis

In the experiment, 40 plants were randomly attributed to each treatment. Percentage root colonization was analysed by one-way ANOVA using Statgraphics Plus for Windows 5.1 software. For the rest of the data, both factors (substrate and mycorrhizal inoculation) and their interaction were analyzed for each parameter by two-way ANOVA, separately in each phase. Ratio and percentage data were subjected to an arcsine square-root transformation before statistical analysis to ensure homogeneity of variance. Treatment means were separated with Duncan's multiple range test ($P \leq 0.05$).

3. Results

3.1. Mycorrhizal colonization

In both substrates, inoculated plants presented a high percentage of root colonization, oscillating between 33% and 65% (Table 1). The plants growing in commercial substrate (C) had the highest values under water stress conditions and the lowest for the well-watered plants. In compost, not significant differences between well-watered and water stressed plants were observed (Table 1).

Table 1. Percentage of mycorrhizal root colonization at the end of the experiment. Values are means ± SEM (n=3 plants). Means within a row without a common lowercase letter are significantly different by Duncan$_{0.05}$ test. P, probability level: ***P≤0.001.

Mycorrhizal inoculation	***Treatments***				***P***
	CM	**CMD**	**CpM**	**CpMD**	
% Colonization	33.40 ± 0.65 c	64.50 ± 1.41 a	56.25 ± 1.52 ab	44.50 ± 0.82 bc	***

3.2. Substrate and leaf mineral content

The mineral content present in the substrates is shown at the end of each phase in Table 2. In phase I, the compost substrate had the highest values for B, K, P and Zn, while the highest levels of Ca and S were found in the commercial substrate. The mycorrhiza effect induced significant increase in Ca content and decrease in P content. When the plants were submitted to water stress (phase II), in compost substrate, the B, Cu, K, P and Zn contents were greater than in commercial substrate, on the contrary occurred for Ca and S contents (Table 2). Mycorrhizae decreased Cu, K, Na, P, S and Zn and increased Ca contents. The interaction between both the substrate and micorrhizae (SxM) was only statically significant for P in phase II (Table 2). The P content increased in the compost substrate and the greatest P value was found in non-mycorrhized plants under water stress conditions.

Table 2. Effects of substrate (S), *G. iranicum* var. *tenuihypharum* inoculation (M) and their interaction (SxM) on the mineral content of the substrate at the end of the first phase (Phase I) and second phase (Phase II). Values are means of 3 samples. *, **, ***, and ns indicate the level of significance at P≤0.05, 0.01, 0.001 and the absence of significance, respectively, according to Duncan's multiple range test.

Substrate mineral content (mg kg^{-1})	***PHASE I***						
	Substrate (S)		**Mycorrhizal inoculation (M)**		**Significance**		
	C	**Cp**	**+**	**-**	**S**	**M**	**SxM**
Cl	7454	7589	7783	7263	ns	ns	ns
B	51.7	65.2	56.6	60.3	*	ns	ns
Ca	22661	19852	22364	20153	**	*	ns
Cu	23.5	28.6	24.3	27.8	ns	ns	ns
Fe	4454	4540	4651	4344	ns	ns	ns
K	4484	6133	5501	5118	***	ns	ns
Mg	4788	4929	4853	4865	ns	ns	ns
Mn	80.8	82.1	83.4	79.6	ns	ns	ns
Na	9776	10208	10208	9691	ns	ns	ns
P	500	638	526	612	**	*	ns
S	10584	8723	9515	9896	*	ns	ns
Zn	37.2	58.0	45.3	50.0	***	ns	ns

Substrate mineral content (mg kg^{-1})	***PHASE II***						
	Substrate (S)		**Mycorrhizal inoculation (M)**		**Significance**		
	C	**Cp**	**+**	**-**	**S**	**M**	**SxM**
Cl	8028	8459	7882	8616	ns	ns	ns
B	43.2	59.4	51.3	51.3	***	ns	ns
Ca	22531	18200	20739	19996	***	*	ns
Cu	21.3	25.7	21.5	25.5	*	*	ns
Fe	4193	3734	4114	3815	ns	ns	ns
K	4510	6128	4888	5753	***	*	ns
Mg	4884	4836	4748	4973	ns	ns	ns
Mn	76.6	82.3	78.9	79.0	ns	ns	ns
Na	7478	8068	7150	8401	ns	*	ns
P	551	914	636.6	829	***	**	*
S	9818	8101	8255	9669	**	**	ns
Zn	42.6	105.2	62.9	85.0	***	*	ns

Plants grown in compost showed the highest levels of Cl, K, Na, P and Zn in leaf in both phases (Table 3). Mycorrhizae mostly affected P content in phase I and Zn levels in phase II, and significant interaction between both factors (SxM) was observed for P in both phases. There was a tendency for P levels to increase in plants growing in compost and for mycorrhizae to have a positive effect on well-watered plants.

Table 3. Effects of substrate (S), *G. iranicum* var. *tenuihypharum* inoculation (M) and their interaction (SxM) on the leaf mineral content of *C. albidus* plants at the end of the first phase (Phase I) and second phase (Phase II). Values are means of 5 plants. *, **, ***, and ns indicate the level of significance at $P \leq 0.05$, 0.01, 0.001 and the absence of significance, respectively, according to Duncan's multiple range test.

Leaf mineral content (mg kg^{-1})	***PHASE I***						
	Substrate (S)		**Mycorrhizal inoculation (M)**		**Significance**		
	C	**Cp**	**+**	**-**	**S**	**M**	**SxM**
Cl	9917	11388	11076	10236	*	ns	ns
Cu	4.64	4.97	5.00	4.61	ns	ns	ns
K	11836	15314	13527	13627	***	ns	ns
Na	1973	2817	2506	2286	*	ns	ns
P	2850	4593	4391	3054	***	***	*
Zn	52.21	64.49	59.65	57.06	**	ns	ns
Leaf mineral content (mg kg^{-1})	***PHASE II***						
	Substrate (S)		**Mycorrhizal inoculation (M)**		**Significance**		
	C	**Cp**	**+**	**-**	**S**	**M**	**SxM**
Cl	9218	12911	10731	11413	*	ns	ns
Cu	3.94	4.20	4.26	3.89	ns	ns	ns
K	12094	15109	14112	13102	*	ns	ns
Na	2606	3734	3023	3322	*	ns	ns
P	2283	3880	3028	3136	***	ns	*
Zn	49.15	73.23	55.85	66.57	***	*	ns

3.3. Plant biomass distribution

At the end of both experimental phases, the leaf fresh weight was lower in plants grown in compost substrate than in plants grown in commercial substrate (Table 4). Under water stress conditions, root fresh weight was 28% lower in the former plants. The decrease in biomass (dry weight) in plants grown in compost was significant for leaves in both phases and for roots in phase II. Mycorrhizae effect and the interaction between both factors (SxM) were not statistically significant (Table 4).

Table 4. Effects of substrate (S), *G. iranicum* var. *tenuihypharum* inoculation (M) and their interaction (SxM) on the fresh and dry weight of leaf, stem and root of *C. albidus* plants at the end of the first phase (Phase I) and second phase (Phase II). Values are means of 3 plants. *, **, ***, and ns indicate the level of significance at P≤0.05, 0.01, 0.001 and the absence of significance, respectively, according to Duncan's multiple range test.

	PHASE I						
	Substrate (S)		**Mycorrhizal inoculation (M)**		**Significance**		
	C	**Cp**	**+**	**-**	**S**	**M**	**SxM**
Fresh weight (g plant^{-1})							
Leaf	5.603	2.570	4.700	3.476	***	ns	ns
Stem	5.829	5.334	5.870	5.297	ns	ns	ns
Root	13.551	10.926	10.903	13.586	ns	ns	ns
Dry weight (g plant^{-1})							
Leaf	2.095	0.934	1.755	1.276	***	ns	ns
Stem	3.122	2.920	3.086	2.958	ns	ns	ns
Root	2.525	1.953	2.177	2.302	ns	ns	ns
	PHASE II						
	Substrate (S)		**Mycorrhizal inoculation (M)**		**Significance**		
	C	**Cp**	**+**	**-**	**S**	**M**	**SxM**
Fresh weight (g plant^{-1})							
Leaf	5.174	2.690	4.226	3.645	***	ns	ns
Stem	5.685	5.744	5.451	5.987	ns	ns	ns
Root	10.935	7.836	8.847	9.941	*	ns	ns
Dry weight (g plant^{-1})							
Leaf	2.114	1.029	1.774	1.372	***	ns	ns
Stem	3.059	3.100	2.943	3.219	ns	ns	ns
Root	2.929	1.915	2.355	2.492	**	ns	ns

3.4. Water status and osmotic adjustment

When all plants were well-irrigated (phase I), leaf water potential (Ψ_l) values were similar in all treatments (Figure 1a and 1b). When water stress was imposed (phase II), a significant drop in Ψ_l levels occurred in the stressed plants, regardless of the treatment applied (substrate or mycorrhiza). Well-watered plants maintained Ψ_l values around -1.0 MPa, similar to those of phase I. In phase II, plants growing in the commercial substrate and submitted to water stress always showed higher Ψ_l values with respect to plants growing in the compost substrate, with a difference of approximately 0.5 MPa (Fig 1). Higher Ψ_l values were observed in inoculated plants at the end of phase II (Figures 1a and 1b). As regards osmotic potential at full turgor (Ψ_{100s}), a decrease was observed in plants grown in compost substrate. Furthermore, this effect was more significant as the experiment progressed (phase II), indicating leaf osmotic adjustment in the plants growing in this substrate (of around 0.45 MPa) (Figures 2a and 2b). Mycorrhiza inoculation did not have a significant effect on Ψ_{100s} when water stress was imposed, and neither the combined effect of the two variables.

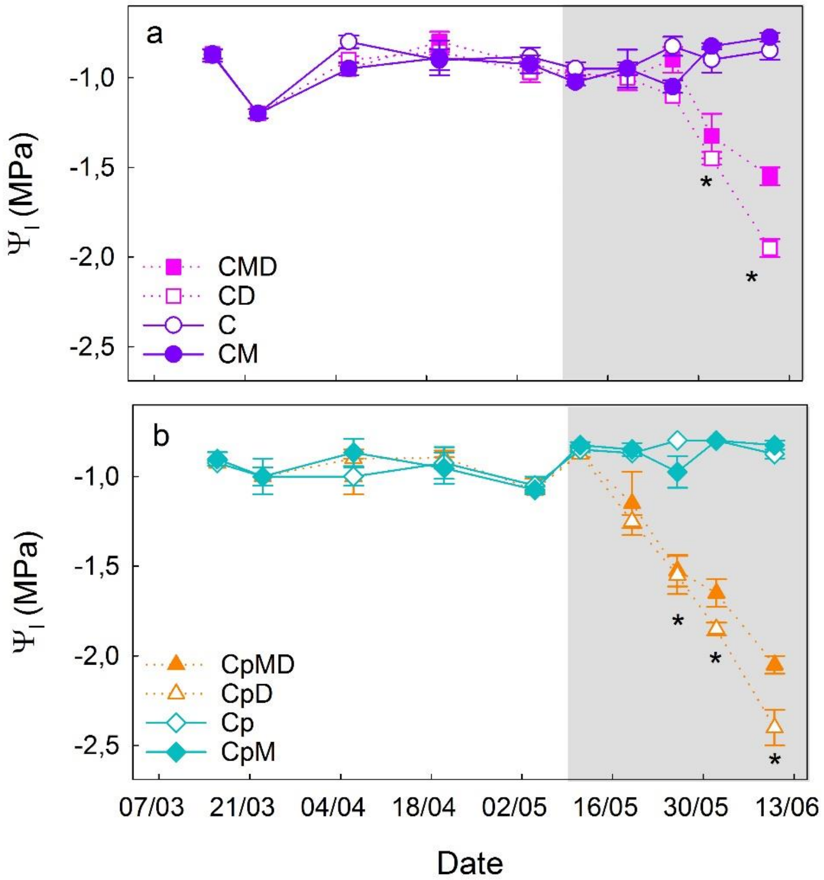

Figure 1. Leaf water potential (Ψ_l) of Cistus albidus plants, grown on commercial (C, a) and compost (Cp, b) substrates, with (M) and without G. iranicum var. tenuihypharum inoculation, and with (D) and without water stress, throughout the experiment. Values are means of four plants. Asterisks indicate statistically significant differences between treatments by Duncan0.05 test. The vertical bars indicate standard errors.

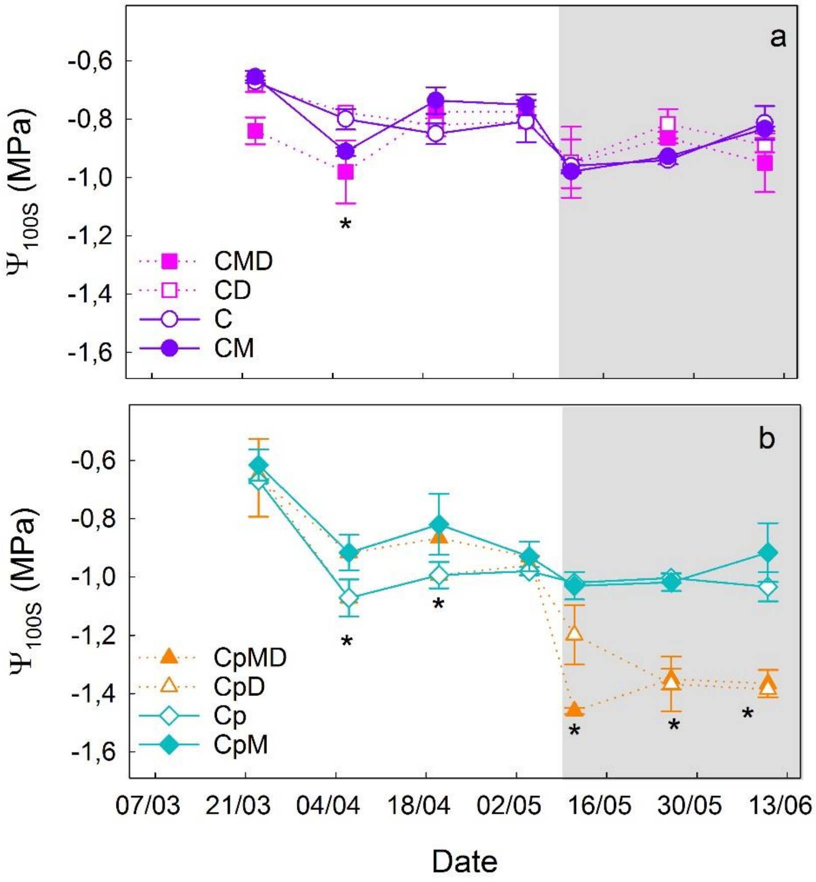

Figure 2. Osmotic water potential at full turgor (Ψ_{100s}) of Cistus albidus plants, grown on commercial (C, a) and compost (Cp, b) substrates, with (M) and without G. iranicum var. tenuihypharum inoculation, and with (D) and without water stress, throughout the experiment. Values are means of four plants. Asterisks indicate statistically significant differences between treatments by Duncan0.05 test. The vertical bars indicate standard errors.

3.5. Gas exchange, leaf temperature and photosynthetic parameters

The substrate did not have a significant effect on stomatal conductance (g_s) during phase I. Throughout phase II, however, g_s values were slightly higher in plants grown in commercial substrate (Figures 3a and 3b). Mycorrhiza inoculation effect and the interaction S x M throughout the whole experiment were not significant.

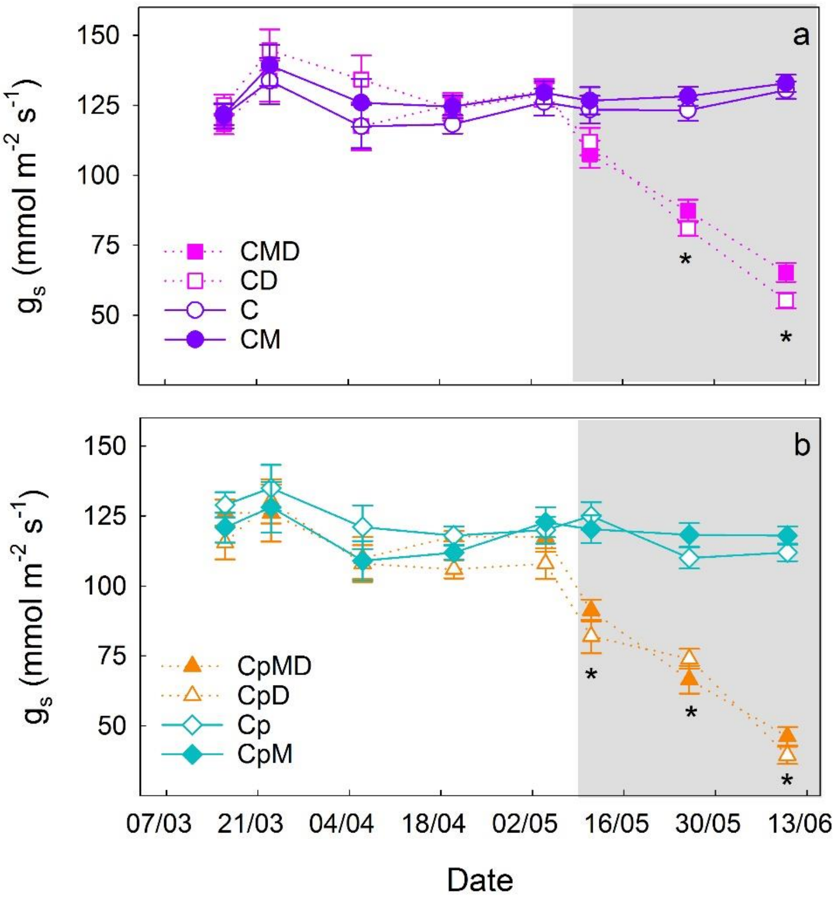

Figure 3. Stomatal conductance (gs) of Cistus albidus plants, grown on commercial (C, a) and compost (Cp, b) substrates, with (M) and without G. iranicum var. tenuihypharum inoculation, and with (D) and without water stress, throughout the experiment. Values are means of four plants. Asterisks indicate statistically significant differences between treatments by Duncan0.05 test. The vertical bars indicate standard errors.

The behavior of P_n tended to be similar to that of g_s (Figures 4a and 4b), although the differences due to substrate were more pronounced than those found in g_s.

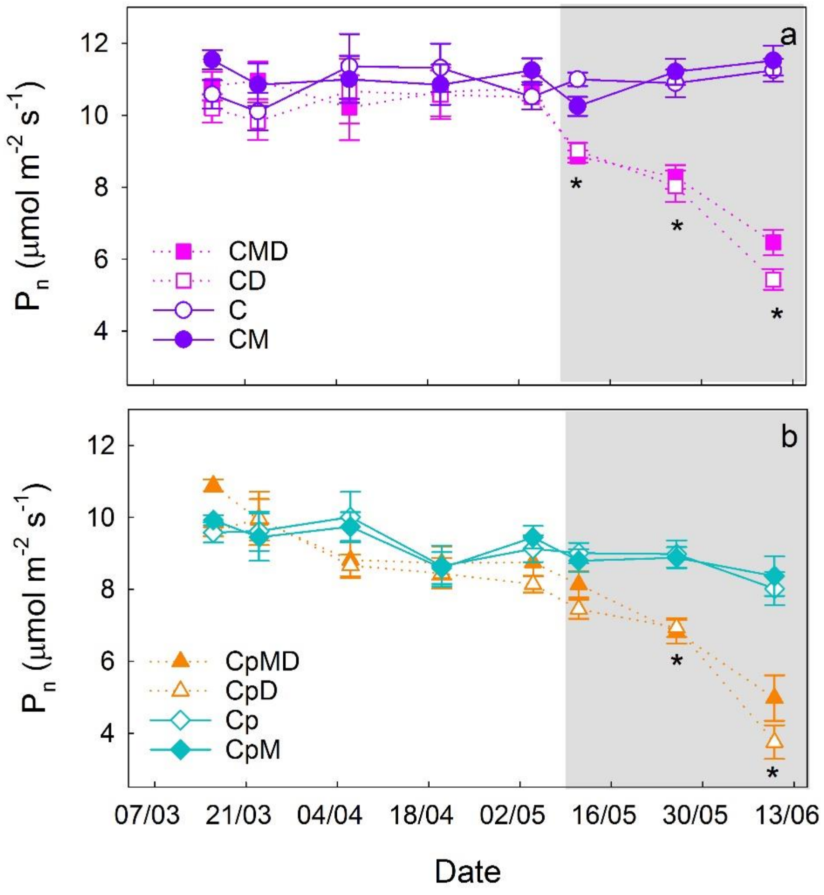

Figure 4. Net photosynthetic rate (Pn) of Cistus albidus plants, grown on commercial (C, a) and compost (Cp, b) substrates, with (M) and without G. iranicum var. tenuihypharum inoculation, and with (D) and without water stress, throughout the experiment. Values are means of four plants. Asterisks indicate statistically significant differences between treatments by Duncan0.05 test. The vertical bars indicate standard errors.

The substrate had a significant effect on leaf temperature, with values that were 2°C greater in plants growing in compost. Mycorrhizal plants in this substrate had lower leaf temperatures than non-inoculated plants at the end of the experiment (Figures 5a and 5b).

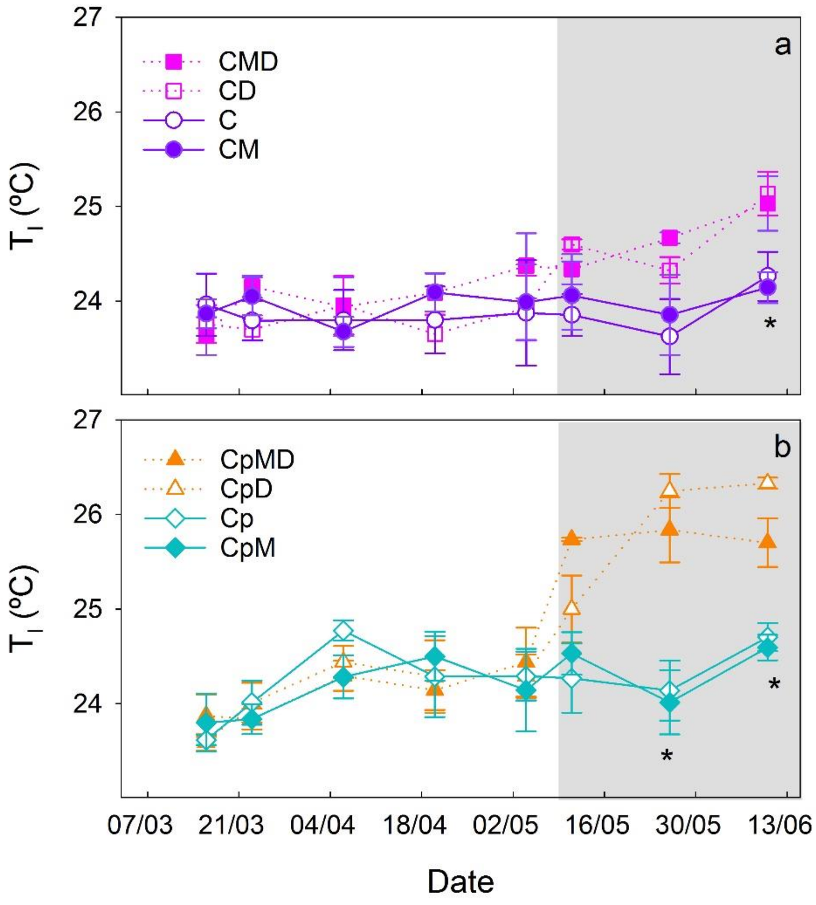

Figure 5. Leaf temperature (T$_l$) of Cistus albidus plants, grown on commercial (C, a) and compost (Cp, b) substrates, with (M) and without G. iranicum var. tenuihypharum inoculation, and with (D) and without water stress, throughout the experiment. Values are means of five plants. Asterisks indicate statistically significant differences between treatments by Duncan0.05 test. The vertical bars indicate standard errors.

In phase I, plants grown in commercial substrate presented higher F_v/F_m and Y(II) values than plants grown in compost. In addition, mycorrhizal inoculation increased the qP parameter and plants growing in compost had higher non-photochemical quenching parameters values [qN, NPQ, Y(NPQ)] (Table 5). In phase II, in the presence of mycorrhizae Fv/Fm, Y(II) and qP increased in commercial substrate (Table 5). Regarding the non-photochemical quenching parameters, plants grown in commercial substrate had higher values than plants grown in compost. In mycorrhizal plants, the non-photochemical quenching parameters increased in plants grown in compost but generally decreased in plants grown in commercial substrate (Table 5).

Table 5. Effects of substrate (S), *G. iranicum* var. *tenuihypharum* inoculation (M) and their interaction (SxM) on chlorophyll fluorescence of *C. albidus* leaves at the end of the first phase (Phase I) and second phase (Phase II). Values are means of 3 plants. Means within a row without a common lowercase letter are significantly different by Duncan$_{0.05}$ test. P, probability level: * P≤0.05; ***P≤0.001.

Chlorophyll fluorescence	***PHASE I***				
	C	**CM**	**Cp**	**CpM**	**P**
Fv/Fm	0.824 ± 0.002 a	0.825 ± 0.002 a	0.775 ± 0.002 b	0.730 ± 0.001 c	***
Y (II)	0.498 ± 0.001 a	0.546 ± 0.002 a	0.448 ± 0.001 a	0.454 ± 0.003 a	ns
qP	0.669 ± 0.002 a	0.72 5± 0.002 a	0.689 ± 0.001 a	0.741 ± 0.001 a	ns
qN	0.452 ± 0.002 ab	0.416 ± 0.002 a	0.570 ± 0.001 c	0.532 ± 0.001 bc	*
NPQ/4	0.154 ± 0.002 ab	0.136 ± 0.001 a	0.222 ± 0.001 b	0.175 ± 0.002 ab	*
Y (NPQ)	0.192 ± 0.002 a	0.161 ± 0.001 a	0.259 ± 0.001 a	0.224 ± 0.002 a	ns

Chlorophyll fluorescence	***PHASE II***				
	C	**CM**	**Cp**	**CpM**	**P**
Fv/Fm	0.769 ± 0.002 a	0.809 ± 0.002 b	0.809 ± 0.001 a	0.752 ± 0.002 a	*
Y (II)	0.460 ± 0.001 a	0.541 ± 0.002 a	0.575 ± 0.001 a	0.444 ± 0.001 a	ns
qP	0.699 ± 0.003 a	0.760 ± 0.001 a	0.786 ± 0.003 a	0.737 ± 0.003 a	ns
qN	0.484 ± 0.001 ab	0.625 ± 0.001 a	0.523 ± 0.002 a	0.501 ± 0.001 b	*
NPQ/4	0.181 ± 0.004 ab	0.182 ± 0.001 ab	0.173 ± 0.002 a	0.253 ± 0.003 b	*
Y (NPQ)	0.226 ± 0.003 a	0.194 ± 0.002 a	0.226 ± 0.002 a	0.281 ± 0.002 a	ns

3.6. Relative chlorophyll content

Plants grown in compost showed a significant decrease in relative chlorophyll content (RCC) in both phases (Table 6). The effect of inoculation (M) increased RCC levels at the end of experiment. The interaction between both factors (SxM) was not significant.

Table 6. Effects of substrate (S), *G. iranicum* var. *tenuihypharum* inoculation (M) and their interaction (SxM) on relative chlorophyll content of *C. albidus* leaves throughout the first phase (Phase I) and the second phase (Phase II). Values are means of 5 plants. *, ***, and ns indicate the level of significance at P≤0.05, 0.001 and the absence of significance, respectively, according to Duncan's multiple range test.

Relative chlorophyll content (%)	***PHASE I***						
	Substrate (S)		**Mycorrhizal inoculation (M)**		**Significance**		
	C	**Cp**	**+**	**-**	**S**	**M**	**SxM**
16/03/2016	35.17	31.77	33.63	33.31	*	ns	ns
23/03/2016	35.81	34.16	35.11	34.87	*	ns	ns
06/04/2016	37.72	31.33	34.21	34.84	***	ns	ns
20/04/2016	36.79	32.47	34.02	35.25	*	ns	ns
04/05/2016	37.14	30.18	34.08	33.24	***	ns	ns
Relative chlorophyll content (%)	***PHASE II***						
	Substrate (S)		**Mycorrhizal inoculation (M)**		**Significance**		
	C	**Cp**	**+**	**-**	**S**	**M**	**SxM**
11/05/2016	35.20	33.31	34.51	34.01	*	ns	ns
26/05/2016	37.01	33.34	35.65	34.72	*	ns	ns
10/06/2016	36.88	30.83	34.73	32.99	*	*	ns

3.7. Lipid peroxidation

In well-watered plants (phase I), there were no significant differences in lipid peroxidation values due to the substrate or inoculation (Table 7). In contrast, when water stress was imposed, higher lipid peroxidation values were found in plants grown in compost than in plants grown in commercial substrate. In mycorrhizal plants, the lipid peroxidation values were lower in both substrates (Table 7).

Table 7. Effects of substrate (S), *G. iranicum* var. *tenuihypharum* inoculation (M) and their interaction (SxM) on lipid peroxidation of *C. albidus* plants at the end of the first phase (Phase I) and the second phase (Phase II). Values are means of 3 plants. * and ns indicate the level of significance at P≤0.05 and the absence of significance, respectively, according to Duncan's multiple range test.

	PHASE I						
	Substrate (S)		**Mycorrhizal inoculation (M)**		**Significance**		
	C	**Cp**	**+**	**-**	**S**	**M**	**SxM**
Lipid peroxidation	3.258	4.197	3.848	3.607	ns	ns	ns
	PHASE II						
	Substrate (S)		**Mycorrhizal inoculation (M)**		**Significance**		
	C	**Cp**	**+**	**-**	**S**	**M**	**SxM**
	3.500	4.417	3.811	4.106	*	ns	ns

4. Discussion

In the production of ornamental plants, organic substrates made with Sphagnum peat and coconut fiber are frequently used. However, high prices, the consideration of peat as a non-renewable resource and heavy reliance on imported materials have all prompted the search for alternative materials (Abad et al. 2001; Larcher and Scariot 2009). One of these materials is compost, which has begun to be used as a component of growing substrates (García-Gómez et al. 2002; Carmona and Abad 2007).

In our assay, although we had another factor of variation such as the application of mycorrhiza, we wanted to know the benefits and/or negative effects (heavy metals) of using compost alone or combined with mycorrhiza under both well-irrigated and water stress conditions. As regards mycorrhizal colonization, *G. iranicum* var*. tenuihypharum* was well established in Cistus roots, despite the fact that the plants were grown in a limited volume of substrate and that this is a genus that generally forms ectomycorrhizae (Caravaca et al. 2005; Comandini et al. 2006). However, mycorrhizae develop differently when plants are under water stress depending on the composition of the substrate. Perhaps water deficit along with components of the compost substrate, such as certain toxic metals (Cu and Zn) and other elements (Graceson et al. 2014) hindered mycorrhizae colonization. On the other hand, commercial substrate did not interfere with colonization. Limited mycorrhizae resulted in more limited plant development in plants grown in compost, which had smaller leaf and root biomass (lower dry weight) than plants grown in commercial substrate. In terms of the mineral content, although plants grown in compost accumulated more Na, because this element is present in the substrate (in a proportion of 1.8 g kg^{-1}), mycorrhizae minimized this effect especially when the substrate was well irrigated (Talaat and Shawky 2014). Due to its composition (potassium=6.8 g kg^{-1}) compost also seems to provoke a greater accumulation of K in the plants, both under stress conditions and especially when the plants were well irrigated.

The effect produced by the compost on the other leaf mineral content was also significant, particularly in the case of phosphorus and zinc. Furthermore, mycorrhizae that developed in the compost substrate in well-watered conditions caused even greater phosphorus accumulation in the plant. This makes sense given the fact that mycorrhizae generally favor the uptake of water and mineral nutrients, especially phosphorus (Augé 2004; Gómez-Bellot et al. 2015b). Other researchers have shown that mycorrhizae (especially arbuscular mycorrhizae) improve phosphorus absorption in arid conditions (Caravaca et al. 2005). The greater and more rapid nutrients availability in the vascular system of plant by the mycorrhizae can accelerates photosynthetic activity, maintaining its physiological balance (Sánchez-Blanco et al. 2009).

On the other hand, although the compost produced higher leaf Na contents, this was not followed by a decrease in K accumulation but rather the opposite. These results demonstrate that compost was not especially harmful, but helped maintain a nutritional balance in plants. Adequate K levels are essential both for plant growth and gas exchange processes (Acosta-Motos et al. 2017). According to Medeiros et al. (2007), crops that generally use N slurry is more efficient than when mineral fertilizers is used. Traditionally has been suggested that the water relations of mycorrhizal plants are influenced by a nutritional effect, linked to the phosphorus (Augé 2001). However, other studies have shown that water relations and gas exchange in mycorrhizal plants could be affected independently of phosphorus nutrition (Morte et al. 2001). In our case, as expected, water stress modified the water status of the plant. Other authors have shown the importance of mycorrhizae in the behavior of stomatal conductance and leaf water potential in a number of species (Dell'Amico et al. 2002; Gómez-Bellot et al. 2013b, 2015a; Vicente-Sánchez et al. 2014). Mycorrhization has also been found to improve efficiency in soil water (Gómez-Bellot et al. 2015a,b); contributing to better water absorption (Allen 1982). In our study, higher phosphorus nutrition in compost and mycorrhizal plants helped improve the water status of the plant, as demonstrated by the less negative leaf water potential values. These results suggest that *G. iranicum* could increase water uptake by the roots (Vicente-Sánchez et al. 2014). However, in non-inoculated plants, an excess of metal ions (Cd, Ni and Zn) in the compost could provoke a water deficit in these plants (Rucinska-Sobkowiak 2016).

The leaf water potential values at maximum illumination reflect the combination of several factors such as vapour pressure deficit (VPD) and radiation, water availability in the soil, internal hydraulic conductivity in the plant and stomatal regulation (Sánchez-Blanco et al. 2002). In this sense, the drop in leaf water potential during the second phase was mainly due to the lack of available water in the substrates, since in our experiment, the environmental conditions were established. The leaf water potential values were similar to those found by Sánchez-Blanco et al. (2002) in *C. albidus* under the same conditions. Under water stress, the osmotic potential at maximum saturation decreased as a result of an active accumulation of solutes due to the mineral composition of the compost (higher B, Cu, K, P and Zn content) (Acosta-Motos et al. 2017). According to inorganic ions, have greater quantitative participation in the osmotic adjustment in both leaves and roots. This is particularly true of like K+ followed by Na and Cl. Similarly, changes in potassium concentrations may contribute substantially to osmo-regulation (Shabala and

Cuin 2007) and may occur in concert with changes in sugars and amino acids (Pérez-Pérez et al. 2009). Our results are in line with those obtained by Patakas et al. (2002), who described on the importance of K in the osmotic adjustment of grapevine plants under water stress.

Stomatal closure is another mechanism used by the plants to reduce water loss to confront water stress (Bañón et al. 2011; Gómez-Bellot et al. 2013a, b; Sánchez-Blanco et al. 2002). In our experimental conditions, furthermore, compost added an osmotic component that provoked a stronger response, following with this idea, stomatal conductance was reduced by Zn and Cu in *Phaseolus vulgaris* L. and in *Sorghum bicolor* L. (Kasim 2006, 2007). Furthermore, in addition to increasing by stomatal closure, leaf temperature can also be increased by the culture medium (Maes and Steppe 2012). This occurred in our experiment, where the plants growing in compost were warmer than those growing in commercial substrate due to osmotic effect. Mycorrhizae did not have a clear effect on these parameters.

Chlorophylls fluorescence is a very useful technique in horticultural studies that allows following the process of photosynthesis as well as the physiological state of the plant in general. In our experiment mycorrhizal inoculation minimized the decrease in chlorophyll levels, showing plants with higher ornamental value (Navarro et al. 2008).

Under well-watered conditions, commercial substrate (C) proved to be more suitable than compost for growing ornamental plants. This conclusion is reflected in the higher photochemical quenching parameter and P_n values in the plants grown in commercial substrate. After the mycorrhizal colonization of the plants, increases in Y(II) and qP in both substrates were related to the greater photosynthetic efficiency recorded. Regarding the non-photochemical parameters, the plants grown in the compost substrate presented, in general, higher NPQ and Y(NPQ) values than plants grown in the commercial, probably due to the lower tolerance of the plants to the heavy metals (Cu and Zn).

When water stress was imposed, plants grown on compost substrate responded slightly better than at the beginning. This was, probably due to a process of acclimatization of the plants, which is reflected in the photochemical quenching parameters, but not in the P_n values. Mycorrhizal plants grown in commercial substrate had better photosynthetic efficiency than mycorrhizal plants grown in compost. The higher non-photochemical quenching parameters values observed in leaves from mycorrhizal plants grown in the compost substrate could be related to protection of the chloroplast in order to dissipate excess light energy safely, thus avoiding photoinhibitory damage (Maxwell and Johnson 2000). On the other hand, the higher lipid peroxidation values found in plants grown in compost possibly reflect greater damage to membranes induced by the conditions imposed by this substrate. However, under water stress, the extent of lipid peroxidation was attenuated in mycorrhizal plants, suggesting the protective role of mycorrhizae inoculation under stress conditions. This response correlated with the higher non-photochemical quenching parameters values.

In conclusion, *C. albidus* plants can complete their growth cycle in commercial and compost substrates, and *G. iranicum* var. *tenuihypharum* is compatible with both substrates. Plants growing in compost maintain a good nutritional balance, higher leaf potassium and phosphorus contents and better osmotic regulation. Nevertheless, under water stress conditions, *C. albidus* plants grown in compost substrate seem to suffer more stress than plants grown in commercial substrate, as reflected by the lipid peroxidation values as well as the P_n values. Although the fungus used it is not an ectomycorrhizal, which is generally used in this genus, it nevertheless minimizes the accumulation of Na in the plant and increases the accumulation of K and P. Furthermore, water stress concentrates compost components (osmotic effect) hindering water uptake and thus limiting plant growth and stomatal opening.

Chapter 1.3

Effect of *Pisolithus tinctorious* on physiological and hormonal traits in cistus plants to water deficit: Relationships among water status, photosynthetic activity and plant quality

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Abstract

Cistus species can form ectomycorrhizae and arbuscular mycorrhizal fungus that can bring benefits when plants are under water stress conditions. However, the application of some ectomycorrhizae on the water uptake under drought through physiological traits and hormonal regulation is less known. The experiment was performed during three months in a growth chamber with *Cistus albidus* plants in which the combined effect of the ectomycorrhiza *Pisolithus tinctorious* inoculation and two irrigation treatments (control and water-stressed plants) were applied. Irrigation absence caused significant decrease in aerial growth and tended to decrease soil water potential at the root surface, leading to a decrease in leaf water potential. Under these conditions, the abscisic acid and salicylic acid content increased while the precursor of ethylene decreased. Although the mycorrhization percentages were not high, the inoculation of *P. tinctorious* improved the water status and slightly cushioned the rise in leaf temperature of water-stressed plants. The ectomycorrhiza decreased the scopoletin values in leaves of plants subjected to deficit irrigation, indicating that inoculated plants had been able to synthesize defense mechanisms. Therefore, P*isolithus tinctorious* alleviated some of the harmful effects of water scarcity in cistus plants, being its use a sustainable option in gardening or restoration projects.

Keywords: Ectomycorrhizae; Mechanisms of resistance; Ornamental plants; Phytohormones; Water relations; Water stress.

1. Introduction

Cistus albidus L. is a typical shrub of the Mediterranean climate. They are widely cultivated both for reforestation and for its use in Mediterranean gardening, especially in Xeriscape, where water management for saving this resource is one of the best options. These plants began to be widely used in gardening and landscaping due to their rusticity and attractive blooms.

Cistus albidus responds to water deficit by developing avoidance mechanisms for the regulation of transpiration based on stomatal closure, reduction of the leaf area and epinastia (Sánchez-Blanco et al., 2009). Furthermore, tolerance to water stress can be explained by other functional and structural adaptations of plants, such as osmotic adjustment, changes in the elasticity of the cell wall, and mineral and hormonal balance (Álvarez et al., 2018).

It is widely known that mycorrhizae confer physiological characteristics to plants that help them improve their water and nutritional status, especially in adverse conditions such as water stress. However, the response of these plants will depend on both the species of the plant and the type of fungus with which it is inoculated, as well as the extension, duration, and type of stress (Ruíz-Lozano et al., 1995; Mardukhi et al., 2011).

Cistus species can form both ectomycorrhizae (ECM) and arbuscular mycorrhizal fungus (AMF); they are dualistic plants (Smith and Read, 2008; Comandini et al., 2006; Caravaca et al., 2005). The dual mycorrhization state is also present in other species such as Populus, Salix, Eucalyptus (Smith and Read, 2008; Comandini et al., 2006). In an earlier experiment we studied whether an AMF, Glomus iranicum var. Tenuihypharum, despite being a genus (Cistus) that generally forms ectomycorrhizae, could bring benefits, especially when plants are under water stress conditions. In that experiment, mycorrhizae helped improve the water status of the plant when irrigation was removed, as demonstrated by the less negative leaf water potential values; it also minimized the decrease in chlorophyll levels, showing plants with higher ornamental value and increased in quantum yield (Y(II)) and photochemical quenching (qP) were related to the greater photosynthetic efficiency recorded (Ortuño et al., 2018).

Several studies have shown beneficial effects of ectomycorrhizal symbioses on the performance of tree species under drought stress (Lehto and Zwiazek, 2011). For example, seedlings of Picea colonized by ECM fungi exhibited increased stomatal conductance and photosynthesis, shoot water potential and growth compared to non-mycorrhizal plants (Lehto, 1992). These ameliorative effects have, at least partly, been ascribed to the increasing in the absorbing surface area and the exploration of larger soil volumes by the extramatrical mycelia, as well as to the role of aquaporins which function is related to post-translational regulation and to the coordination of the phytohormones (Navarro-Ródenas et al., 2013; Xu et al., 2015). Transport of water and nutrient uptake through hyphae to host plants improves plant water and nutrient supply improving the performance of plants during periods of drought stress (Lehto and Zwiazek, 2011). Furthermore, drought-stressed ECM-colonized plants show increased hydraulic conductance compared with non-mycorrhizal plants (Mushin and Zwiazek, 2002; Bogeat-Triboulot et al., 2004). Concretely, Pisolithus tinctorius is one of the most widespread ECM fungi and can establish associations with a broad variety of species and has been recorded in a wide range of habitats (Carney, 1997). P. tinctorius has great mycorrhizal capacity and provides the plant with greater efficiency in the absorption of water and nutrients (Marx and Cordell, 1989), so it has been used in reforestation program sand and in commercial ECM inoculum production (Sebastiana et al., 2013).

In general, mycorrhizal fungi plays an important role in regulating phytohormones such as jasmonic acid (JA), salicylic acid (SA), and ethylene (ET), which are involved in the host defense response, and abscisic acid (ABA), gibberellin (GA), and citokinin (CK), which modulate the growth of the plants. However, while the role of hormones in AMF-inoculated plants is widely known (Quiroga et al., 2018; Sánchez-Romera et al., 2018), less is known in ECM-inoculated plants, although, fundamentally, studies of the effect of ABA and SA in ECM-inoculated plants under adverse conditions have been carry out. For example, Rincón et al. (2005) evaluated whether two ectomycorrhizal fungi (*Laccaria bicolorthe* and *Cenococcum geophilum*) affected ABA production in larch during osmotic stress and Luo et al. (2009) determined the phytohormone changes in plants inoculated with *Paxillus involutus* under salinity. However, in the case of P. tinctorius, the relationships between water status and hormonal production in plants subjected to water stress is less known.

For all the above, in this study we examine how the interaction of *Cistus albidus* with the ectomycorrhizal fungus *Pisolithus tinctorious* affects water uptake under drought through physiological traits and hormonal regulation. The objectives of the study were (i) to study physiological and hormonal response to water deficit; (ii) to evaluate the effect of P. tinctorious on physiological traits and hormonal regulation of Cistus plants; and (iii) to analyze the changes in the relationships among physiological, morphological, and ornamental parameters under water deficit and mycorrhizal inoculation. Comparative data relating these physiological processes to water stress may prove beneficial toward understanding the drought tolerance of plants.

2. Material and Methods

2.1. Plant material and experimental conditions

Forty-eight plants of *Cistus albidus* L. from the nursery were used. On January 18, 2017, these plants were transplanted into 1.5 L pots with a commercial substrate based on Sphagnum peat, coconut fiber and perlite in an 8:7:1 ratio and were placed in a controlled growth chamber. The climatic conditions of the chamber were those necessary for optimal growth: temperature (23ºC/18ºC, day/night), photosynthetic photon flux density (350 µmol m^{-2} s^{-1}), photoperiod (16h/8h, light/dark), and relative humidity (RH) (60%). Plants were watered at field capacity.

2.2. Treatments

After two weeks of acclimatization, half of the plants (24) were inoculated with the ectomycorrhizal fungus *Pisolithus tinctorius*. Two months later, once the plants were stabilized, the irrigation of half of inoculated and non-inoculated plants were removed for a month, while the remaining plants continued to be irrigated at field capacity. Thus, the following treatments were established: C, Control, well-watered plants; CM, well-watered and inoculated plants; WS, plants subjected to withholding; WSM, inoculated plants subjected to water stress. Each treatment included three replications. Each replication consisted of four plants. The experiment lasted three months.

2.3. Fungal colonization

At the end of the experiment, roots samples with the surrounding rhizosphere soil were collected to treat to evaluate fungal development. Three root samples were used in each replication. The percentage of mycorrhizal root colonization was estimated as following: Once cleaned, the roots were immersed in KOH (100 ºC for 7 min), followed by a bath in H2O2 (100 ºC, 5–6 min) and finally, trypan blue staining (4 min). The percentage of colonization was calculated using the methodology proposed by Kormanik and McGraw (1982). The colored roots were placed on specialized plates for counting and were observed under the magnifying glass by counting 100 fields neatly (positive and negative). The percentage of colonization was calculated using the following formula proposed by Sieverding (1983):

$$\%\,colonization = \frac{number\ of\ colonized\ fields}{total\ number\ of\ fields\ observed} \times 100$$

Picture 1. Root staining process (left) and Petri dish for counting (right).

2.4. Leaf mineral content

At the end of the experimental period, the inorganic mineral content of dry leaves was determined in three plants per replication by means of emission spectrophotometry. The nutrient concentrations were determined in a digestion extract with HNO_3:$HClO_4$ (2:1, v/v) by Inductively Coupled Plasma optical emission spectrometer (ICP-OES IRIS INTREPID II XDL, Thermo Fisher Scientific Inc., Loughborough, UK).

2.5. Biomass and height

At the end of the experiment, three plants per replication were selected and all the substrate was gently washed from their roots. After washing the substrate from the roots, the plants were individually separated into leaves, stems and roots and the fresh weight of each organ was determined. After that, each sample was dried in an oven at 80 ºC, until samples reached a constant weight, and the dry weights (DW) were obtained.

Every 15 days, the height of three plants per replication was measured. Plant height was measured from the base of the plant at the substrate surface to the most distal growth.

2.6. Water relations

Leaf water potential (Ψ_l), leaf osmotic potential (Ψ_s) and osmotic water potential at full turgor (Ψ_{100s}) were determined in three plants per replication throughout the experimental period. The leaf water potential was determined during light hours according to the technique described by Scholander et al. (1965), using a pressure chamber (Model 3000; Soil Moisture Equipment Co., Santa Barbara, CA, USA), the leaves were pressurized at a rate of 0.03 MPa s^{-1}. Ψ_s and Ψ_{100s} was measured using a Wescor 5520 vapor pressure osmometer (Wescor Inc., Logan, UT, USA), according to Gucci et al. (1991). For Ψ_{100s} the leaf samples were previously subjected to a rehydration treatment by dipping their petioles in distilled water for 24 h to achieve complete saturation.

Changes in Ψ_s ($\Delta\Psi_s$) and in Ψ_{100s} ($\Delta\Psi_{100s}$) were calculated according to Girma and Krieg (1992) as the difference in Ψ_s and Ψ_{100s} measured at the initial (i) and at the end (e) of water stress period.

$$\Delta\Psi_S = (\Psi_S)^i - (\Psi_S)^e$$

$$\Delta\Psi_{100S} = (\Psi_{100S})^i - (\Psi_{100S})^e$$

The contribution of dehydration to changes of Ψ_S ($\Delta\Psi_{SS}$) was calculated using the following equation:

$$\Delta\Psi_{SS} = \Delta\Psi_S - \Delta\Psi_{100S}$$

Soil water potential at the soil-root interface (Ψ_r) was computed according to Jones (1983):

$$\Psi_r = (\Psi_l^{WS} - \Psi_l^{C}) \times g_s^{WS} / g_s^{C}$$

where, Ψ_l WS and Ψ_l C correspond to the mean value of the leaf water potential in the WS and C treatments, respectively, and the gs WS and gs C correspond to the mean value of the respective treatments. A value of Ψ_r was calculated for each of the three drought pots using the value of Ψ_l C and gs C. The Ψ_r is assumed to be zero for control plants.

2.7. Gas exchange and thermography

Stomatal conductance (g_s) and net photosynthesis rate (Pn) were measured with the LICOR 6400 portable meter (LI-COR Inc., Lincoln, NE, USA). The flow rate of circulating air within the system was approximately 200 mmol s^{-1}, with a leaf vapour pressure deficit to air of about 2 KPa. The CO2 concentration was fixed at 380 ppm and the photosynthetically active radiation (PAR) at 600 µmol m^{-2} s^{-1}. The measurements were carried out in three plants per replication on the same days as the water relations.

At the same time and in the same plants that gas exchange was determined, leaf temperature (T_l) was measured with an infrared camera (FLIR-e50 System, Inc., Danderyd, Sweden) which consisted of a 240 × 180 pixels line scan imager operating in the $7.5e^{13}$ mm region, with a noise equivalent temperature difference of 0.05 ºC at 30 ºC and an accuracy of 2 ºC or 2% of the reading. The background temperature, distance of the camera from the canopy, air temperature, emissivity, and relative humidity were used as input at the start of each series of measurements; so, the camera automatically corrects for atmospheric transmission based on these data. Background temperature was determined as the temperature of a crumpled sheet of aluminium foil in a similar position to the leaves of interest with the emissivity set at 1.0. Emissivity for leaf measurements was set at 0.96 (Leinonen et al., 2006) and the distance at which images were taken was 0.5 m. The images were processed with ThermaCam Researcher Professional 2.10 software (FLIR Quick Report, Danderyd, Sweden).

2.8. Relative chlorophyll content

The relative chlorophyll content (RCC) was determined periodically in three plants per replication with a Minolta SPAD-502 chlorophyll meter (Konica Minolta Sensing Inc., Osaka, Japan), a non-destructive method.

2.9. Hormonal determination

The extraction and analysis of the plant hormones were developed in agreement with Albacete et al. (2008) including slight modifications in the protocol, such as the measurement of scopoletin (phytoalexin). Firstly, 100 mg of fresh plant material (leaf) was homogenized in liquid nitrogen and placed in 0.5 mL of cold (−20 ºC) extraction mixture of methanol/water (80/20, v/v). The mix was centrifuged at 15,000 r.p.m. (20,627× g), 4 ºC for 15 min. The supernatants were kept at 4 ºC and the remaining plant material (pellet) was re-extracted with additional 0.5 mL of the same extraction solution. The mix was re-centrifuged at 15,000 r.p.m. (20,627× g), 4 ºC for 15 min. Pooled supernatants were passed through a Chromafix C18 cartridge (previously activated with 3 mL methanol/water (80/20, v/v) to remove interfering lipids and plant pigments and evaporated to dryness (3 h, approximately). The residue was dissolved in 200 μL metanol/water (80/20, v/v), then sonicated during 8 min and filtered through Millex filters with 0.45 μm pore size nylon membrane (Millipore, Bedford, MA, USA). The final sample extracted was injected into an ultra-high-performance liquid chromatography (UHPLC) coupled triple quadrupole mass spectrometry (UHPLC-QqQ-MS/MS).

Picture 2. Hormone extraction process.

The separation of plant hormones and phytoalexins was developed with slight modifications in accordance with Albacete et al. (2008) by using UHPLC coupled to a 6460 triple quadrupole mass spectrometer (Agilent Technologies, Waldbronn, Germany), and a BEH C18 column (2.1 × 50 mm, 1.7 µm) (Waters, Milford, MA, USA) with a guard column (2.1 × 5.0 mm, 1.7 µm). The column temperature was 40 ºC. Water/acetic acid (99.99:0.01, v:v) (solvent A) and acetonitrile (solvent B) were used as mobile phases at the flow rate of 0.2 mL min^{-1}. The injection volume was 10 µL. The gradient program used was: 19% B at 0 min, 90% B at 2.5 min, 90% B at 4.5 min, 19.0% B at 6 min, and 19% B at 8.0 min for column equilibration. The electrospray interface (ESI) was set up in the negative and positive mode and the mass spectrometry analysis were run in the multiple reaction monitoring modes (MRM). The ionization and fragmentation conditions were as follows: gas temperature 325 ºC, gas flow 8 L/min, nebulizer 45 psi, sheath gas temperature 375 ºC, jet stream gas flow 11 L/min, capillary voltage 4000 V and 2750 V (positive and negative mode, respectively), and nozzle voltage 1000 V and 1500 V (positive and negative mode, respectively) according to the most abundant product-ions. The quantitative evaluation of plant hormones and scopoletin was carried out using authentic standards. The standards used are included in Table 5 and were diluted in water/methanol (20:80, v/v) for the quantification curves.

Table 1. Plant hormones and phytoalexins: reference, brand and solubility

Hormone	Reference	Laboratory	Solubility
Jasmonic acid	14631-	SIGMA	DMSO (16
trans-Zeatin	Z0876-5MG	SIGMA	DMSO (3 mg/mL)
trans-Zeatin	001030n (5	OLCHEMLM	DMSO (3 mg/mL)
[2H5]-trans-Zeatin	030030n (1	OLCHEMLM	DMSO (3 mg/mL)
Camalexin	SML1016-	SIGMA	DMSO (20
Isopentenyladenine	SC-279669	SANTA CRUZ	DMSO (20
trans-Zeatin riboside	SC-20846	SANTA CRUZ	ETHANOL/DMSO
trans-Zeatin riboside	001031n (5	OLCHEMLM	DMSO
trans-Zeatin glucoside	SC-237225	SANTA CRUZ	ETHANOL/DMSO
trans-Zeatin 9-glucoside	001047n (1	OLCHEMLM	DMSO
Abscisic acid	SC-238015	SANTA CRUZ	ETHANOL (50
3-indoleacetic acid	SC-254494	SANTA CRUZ	ETHANOL (50
1-aminocyclopropane-1-carboxylic	SC-202393	SANTA CRUZ	WATER (437
Giberellic acid (GA1)	012249n (1	OLCHEMLM	ETHANOL (50
Giberellic acid (GA3)	SC-257556	SANTA CRUZ	ETHANOL (50
Giberellic acid (GA4)	SC-235248	SANTA CRUZ	ETHANOL (50
Giberellic acid (GA5)	SC-490117	SANTA CRUZ	ETHANOL (50
Giberellic acid (GA7)	012254n (1	OLCHEMLM	ETHANOL (50
Salycilic acid	SC-203374	SANTA CRUZ	ETHANOL (50
Scopoletin	SC-206059	SANTA CRUZ	DMSO (30

All the phytohormones were determined in three plants per block at the end of the experimental period.

2.10. Statistical analysis

In the experiment, 48 plants were randomly attributed to each treatment, with three replications for each treatment. The data were analysed by one-way ANOVA using Statistical Package for the Social Sciences (IBM SPSS Statistics 26 for Windows, CA, USA). Treatment means were separated with Duncan’s Multiple Range Test ($P \leq 0.05$). Prior to the statistical analysis, percentage of root colonization was subjected to an arcsine squareroot transformation to ensure the homogeneity of the variance. Relationships between parameters were fitted to different regressions using SigmaPlot v. 14.5 software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Mycorrhization percentage and growth

At the end of the experiment, mycorrhizal inoculation produced a colonization of around 30% in root systems of well-irrigated Cistus plants. The level of colonization in roots of mycorrhizal plants decreased near to 20% with the suppression of the irrigation (Table 1). In both cases, the mycorrhization percentages were not high.

Table 2. Percentage of root colonization in plants subjected to control and water stress with *Pisolithus tinctorious* (CM and WSM, respectively) at the end of the experiment. Values are means of three root samples per replication.

	Mycorrhization percentage (%)
CM	29.3 a
WSM	21.05 b

Different letters indicate significant differences according $\text{Duncan}_{0.05}$ test

Water stress caused significant decrease in aerial growth parameters such as height and leaf DW, being the latter the one that decreases the most (30% respect to C) (Table 2). The inoculation of *Pisolithus tinctorious* increased almost all growth parameters for both irrigation conditions, as observed when we compare CM with C and WSM with WS. Indeed, inoculated plants of both irrigation treatments (CM and WSM) were taller and their leaf and root DW were higher than those corresponding to non-inoculated plants (C and WS) (Table 2).

Table 3. Dry weight of leaf, stem, root and shoot, root/shoot ratio, and height of *Cistus albidus* plants under well-irrigated and non-irrigated conditions, with and without *Pisolithus tinctorious* mycorrhiza, at the end of the experiment. Values are means ± SEM (n = 3).

	TREATMENTS			
	C	**WS**	**CM**	**WSM**
Leaf DW (g)	3.32±0.39b	2.37±0.31c	5.59±1.44a	4.06±0.41ab
Stem DW (g)	2.25±0.30	2.05±0.22	3.58±1.05	2.80±0.15
Root DW (g)	1.64±0.15c	1.55±0.20c	2.05±0.13b	3.17±0.30a
Shoot DW (g)	5.58±0.68bc	4.43±0.54c	8.50±0.89a	6.85±0.55ab
Root/shoot ratio	0.30±0.03b	0.35±0.01ab	0.22±0.03b	0.48±0.09a
Height (cm)	33.42±1.35b	30.50±1.08c	35.83±1.46a	33.23±1.49b

DW, dry weight; C, well-watered plants; WS, non-irrigated plants; CM, well-watered and inocu-lated plants; WSM, non-irrigated and inoculated plants. Different letters in rows indicate significant differences between treatments according Duncan$_{0.05}$ test. Absence of letters in rows indicates no significant difference between treatments.

3.2. Leaf mineral content

Water stress had a significant effect on leaf mineral content decreasing almost the entire mineral measured, when comparing C and WS, having a highly significant effect in Ca, Na, Mg, and Cu. The effect of the mycorrhiza treatment was hardly significant. Under well-watered conditions, only the leaf content of Ca and S in CM plants increased their values both in around 30% respect C plants (Table 3). Under water stress conditions, ECM produced a slight tendency to decrease almost all the elements of WSM plants respect to WS (Table 3).

Table 4. Leaf mineral content of *Cistus albidus* plants under well irrigated and non-irrigated conditions, with and without *Pisolithus tinctorious* mycorrhiza, at the end of the experiment. Values are means ± SEM (n = 3).

	TREATMENTS			
	C	**WS**	**CM**	**WSM**
	Macronutrients			
Ca (%)	0.86 ± 0.02 b	0.60 ± 0.03 c	1.00 ± 0.05 a	0.49 ± 0.05 c
K (%)	3.19 ± 0.23 a	2.72 ± 0.50 ab	3.02 ± 0.32 a	2.18 ± 1.28 b
P (%)	0.67 ± 0.06 ab	0.59 ± 0.03 ab	0.73 ± 0.06 a	0.54 ± 0.04 b
Na (%)	0.14 ± 0.02 a	0.08 ± 0.01 b	0.16 ± 0.01 a	0.05 ± 0.01 b
S (%)	0.17 ± 0.03 b	0.16 ± 0.02 b	0.22 ± 0.01 a	0.15 ± 0.01 b
Mg (%)	0.35 ± 0.02 a	0.26 ± 0.02 b	0.37 ± 0.01 a	0.24 ± 0.01 b
	Micronutrients			
Cu (mg/Kg)	12.95 ± 1.29 a	4.97 ± 0.82 b	10.85 ± 2.23 a	4.15 ± 0.45 b
Fe (mg/Kg)	68.78 ± 7.44 ab	54.68 ± 4.96 b	82.97 ± 10.13 a	102.85±35.34 a
Mn (mg/Kg)	117.13 ± 3.41 ab	123.52 ±28.55 ab	153.05 ± 7.71 b	108.93 ± 23.75a
B (mg/Kg)	165.35 ± 3.29	158.55 ± 1.40	162.75 ± 3.98	161.10 ± 7.69
Zn (mg/Kg)	87.95 ± 13.71 a	59.58 ± 9.09 ab	72.73 ± 5.42 ab	41.97 ± 7.37 b

C, well-watered plants; WS, non-irrigated plants; CM, well-watered and inoculated plants; WSM, non-irrigated and inoculated plants. Different letters in rows indicate significant differences between treatments according $\text{Duncan}_{0.05}$ test. Absence of letters in rows indicates no significant difference between treatments.

3.3. Water relations

Soil water potential at the root surface (Ψ_r) tended to decrease in WS plants due to water stress, causing greater resistance to water absorption, which leads to a decrease in leaf water potential. No differences were observed due to the effect of mycorrhizae (Figure 1A).

In In well-watered plants, both inoculated and non-inoculated (CM and C, respectively), the values of leaf water potential (Ψ_l) remained at −1.0 MPa, showing a good water status of the plants (Figure 1B). When stress was applied, non-inoculated plants (WS) decreased Ψ_l significantly, reaching minimum values of −3.0 MPa. The use of *Pisolithus tinctorius* alleviated significantly the negative effect of water stress and the fall in values was not so severe (−2.1 MPa) (Figure 1B).

Figure 1C shows the contribution to dehydration to change in osmotic water potential ($\Delta\Psi$SS). Under well-watered conditions, $\Delta\Psi$SS was almost negligible for both inoculated and non-inoculated plants (C and CM, respectively). Water stress caused a decrease in $\Delta\Psi$SS, reaching minimum values of around −0.27 MPa in non-inoculated plants (WS), while the ECM dampened this drop (−0.12 MPa).

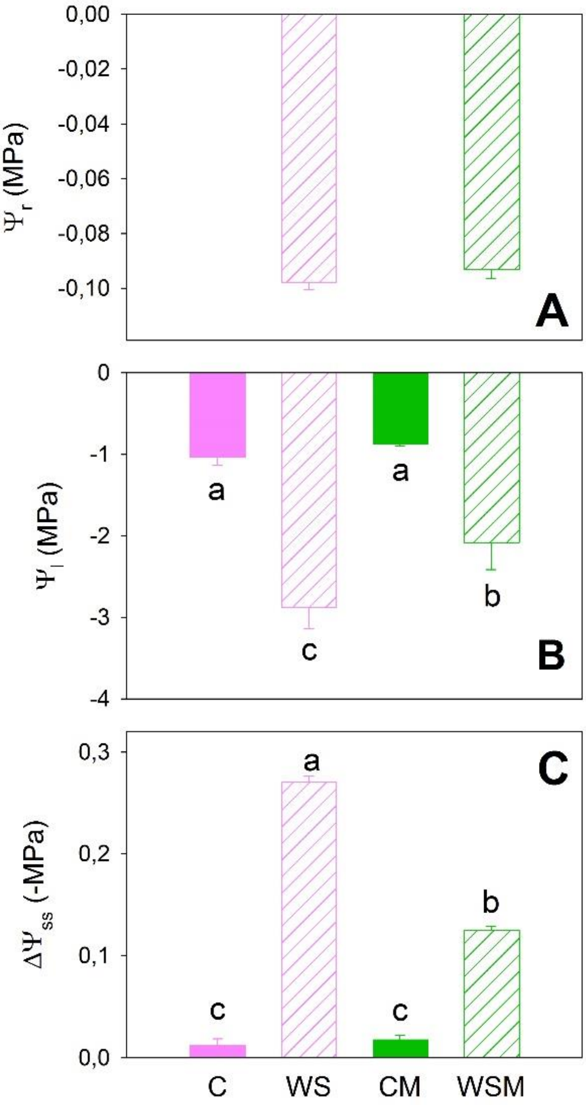

Figure 1. Soil water potential at the root surface (Ψ_r) (A), leaf water potential (Ψ_l) (B) and total seasonal leaf osmotic water potential changes due to dehydration ($\Delta\Psi$SS) (C) of *Cistus albidus* plants under well-irrigated and non-irrigated conditions, with and without *Pisolithus tinctorious* inoculation, at the end of the experiment. Values are means of three replications. The vertical bars indicate standard errors. Different lowercase letters indicate significant differences between treatments according to Duncan$_{0.05}$ test. Absence of letter s in rows indicates no significant difference between treatments. C, well-watered plants; WS, non-irrigated plants; CM, well-watered and inoculated plants; WSM, non-irrigated and inoculated plants.

3.4. Gas exchange

Under water stress conditions, the stomatal conductance (g_s) fell drastically, almost reaching the stomatal closure in WS plants. Differences of 150 mmol m^{-2} s^{-1} between C and WS treatments were observed (Figure 2A). *Pisolithus tinctorious* did not favor opening in either case, neither under conditions of good irrigation nor under water deficit (CM and

WSM). Net photosynthesis (P_n) showed a behavior very similar to that of g_s (Figure 2B). Leaf temperature (T_l) of plants subjected to water stress (WS) was 1.5ºC higher than C plants (Figure 2C).

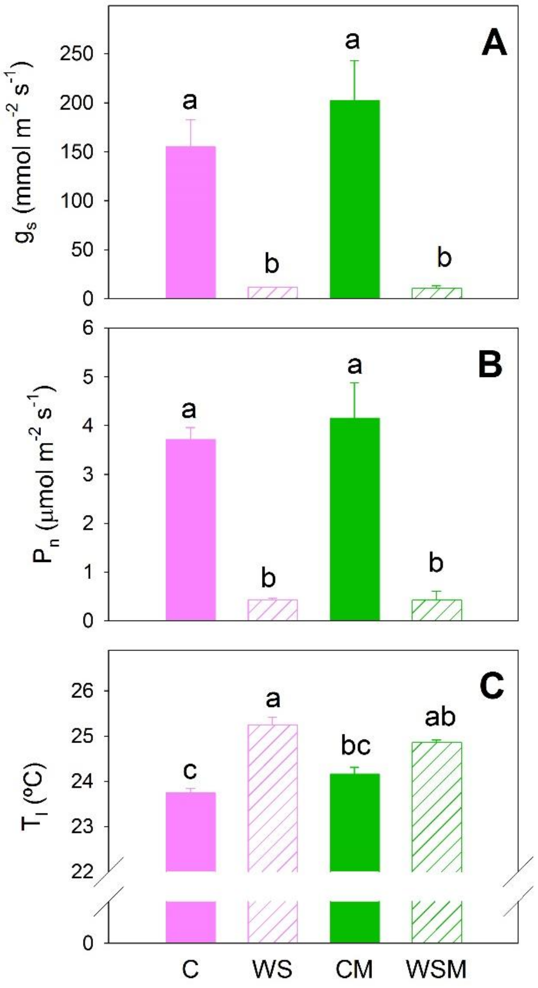

Figure 2. Stomatal conductance (g_s) (A), net photosynthetic rate (P_n) (B) and leaf temperature (T_l) (C) of *Cistus albidus* plants under well-irrigated and non-irrigated conditions, with and without *Pisolithus tinctorious* inoculation, at the end of the experiment. Values are means of three replications. The vertical bars indicate standard errors. Different lowercase letters indicate significant differences between treatments according to Duncan$_{0.05}$ test. C, well-watered plants; WS, non-irrigated plants; CM, well-watered and inoculated plants; WSM, non-irrigated and inoculated plants.

3.5. Phytohormones

At the end of the experiment period, the abscisic acid (ABA) and salicylic acid (SA) content in WS leaves were approximately 5 and 3 times as high as those in control leaves, respectively (Figure 3A,B). By contrast, the precursor of ethylene (ACC) content was decreased significantly by the irrigation suppression (45% of C) (Figure 3C). Mycorrhizae only had a significant effect on scopoletin (SC) under water stress conditions (WSM), significantly decreasing their values by approximately 46% respect to non-inoculated plants (WS) (Figure 3D).

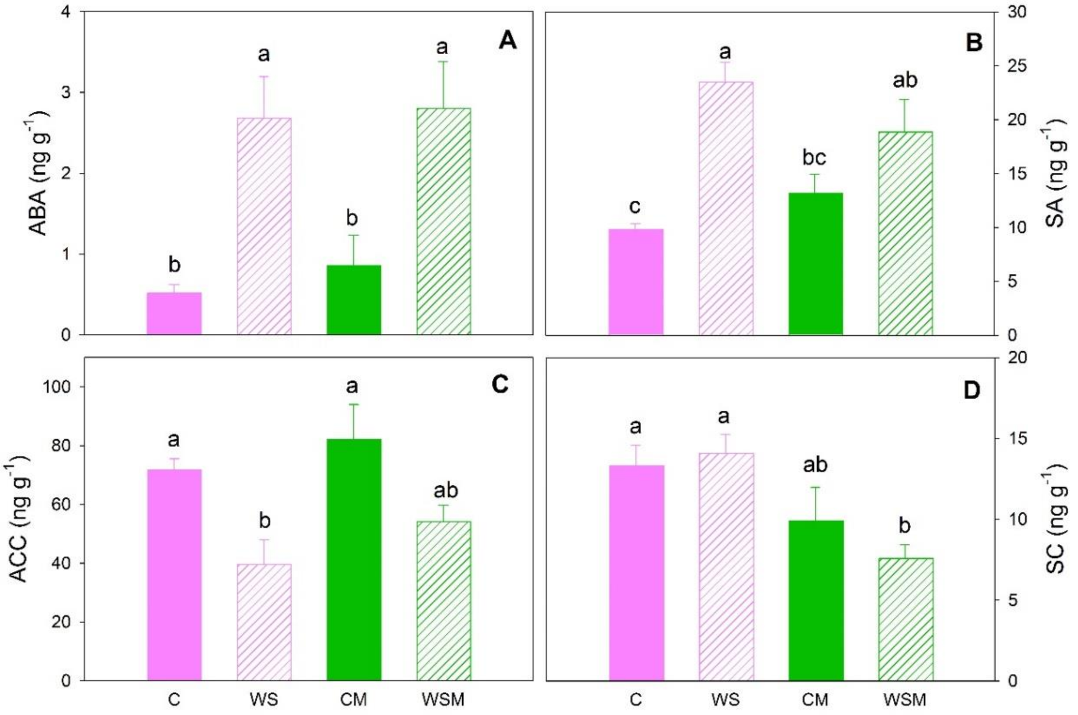

Figure 3. Abscisic acid (ABA) (A), salicylic acid (SA) (B), precursor of ethylene (ACC) (C), and scopoletin (SC) (D) of *Cistus albidus* plants under well-irrigated and non-irrigated conditions, with and without *Pisolithus tinctorious* inoculation, at the end of the experiment. Values are means of three replications. Different lowercase letters indicate significant differences between treatments according to Duncan$_{0.05}$ test. The vertical bars indicate standard errors. C, well-watered plants; WS, nonirrigated plants; CM, well-watered and inoculated plants; WSM, non-irrigated and inoculated plants.

3.6. Relationships between physiological, morphological and ornamental parameters

The relationship between gs and Pn was well represented by an exponential function when all the values of the four treatments obtained during the experimental period were considered (Figure 4A). Highly significant linear regressions between gs versus Pn can be obtained when considering control treatments (C and CM) and water stress treatments (WS and WSM) individually, suggesting that gs explain differences in Pn within each irrigation treatment. A strong correlation (R^2 = 0.86) between gs and leaf water potential

(Ψ_l) was observed, fitting with an exponential sigmoid model. Ψ_l of plants subjected to water stress (WS and WSM) declined sharply (variation between −1.0 and −3.2 MPa) in a narrow range of gs (Figure 4B). Relationship between Tl and gs fits with an exponential decay model. Leaf temperature was maintained between 23.3 and 24.3ºC when the stomatal conductance exceeded approximately 65 mmol m^{-2} s^{-1} (Figure 4C).

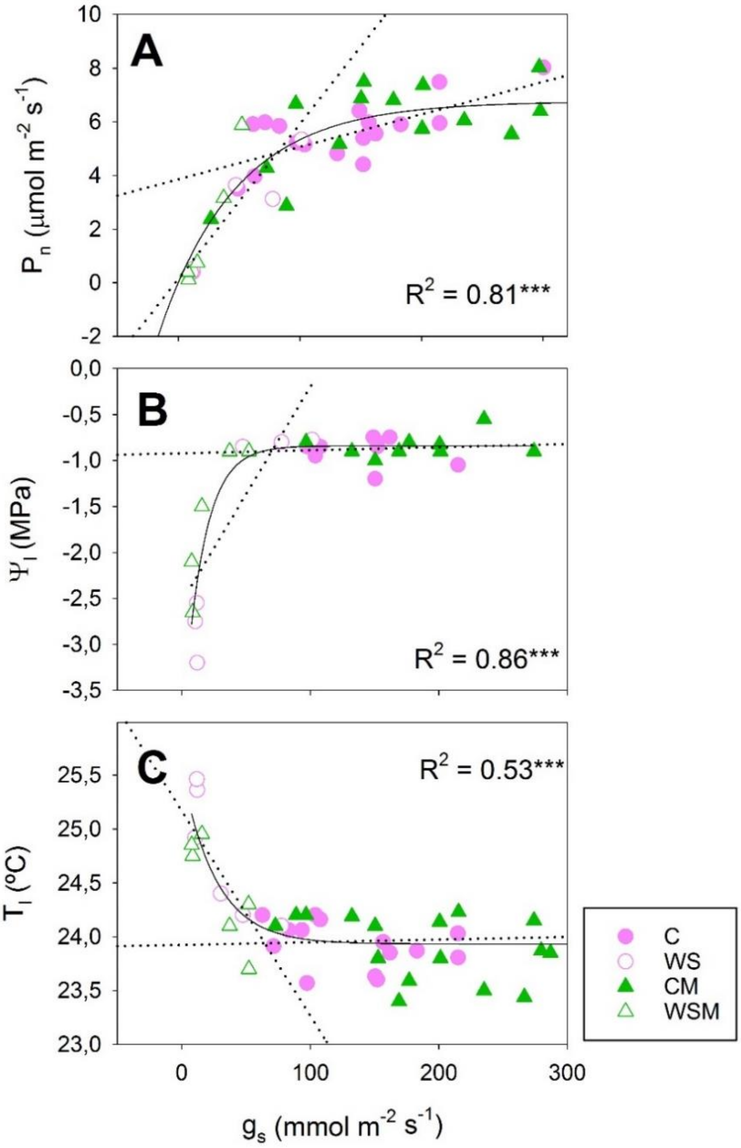

Figure 4. Solid lines represent the relationships between stomatal conductance (gs) and net photosynthesis (P_n) (A), gs and leaf water potential (Ψ_l) (B) and gs and leaf temperature (T_l) (C) in *Cistus albidus* plants under well-irrigated and non-irrigated conditions, with and without *Pisolithus tinctorious* inoculation, during the experimental period. Dotted lines represent the linear regressions of the control (C and CM) and water stress treatments (WS and WSM) individually. Values are means of three replications. C, well-watered plants (closed circles); WS, non-irrigated plants (open circles); CM, well-watered and inoculated plants (closed triangles); WSM, non-irrigated and inoculated plants (open triangles). *** significant at $P < 0.001$.

With increasing water stress, the dependence of Ψ_l and T_l on gs increased, as well as the dependence of the relative chlorophyll content (RCC) on P_n. WS as compared to C promoted intrinsic water use efficiency (WUE, P_n/g_s). However, P_n was less dependent on shoot dry weight when the plants were subjected to irrigation suppression (Table 4). Under water stress conditions, *Pisolithus tinctorious* only had effect on the relation between RCC and P_n, being WSM higher than WS (Table 4).

Table 5. Water indices related to gas exchange and photosynthetic efficiency at the end of the experiment. Values are means ± SEM (n = 3).

	TREATMENTS			
	C	**WS**	**CM**	**WSM**
P_n /g_s (µmol CO_2/ mol H_2O)	26.21± 4.41b	38.58±3.87a	23.31±8.62b	40.55±2.10a
-Ψ_l /g_s (MPa/ mol H_2O m^{-2} s^{-1})	5.40±0.23b	170.10±45.75a	4.73±1.05b	205.97±33.10a
T/g_s (ºC/ mol H_2O m^{-2} s^{-1})	166.2±32.61b	2215.92±82.63a	130.34±27.72b	2576±50.02a
RCC/P_n (%/⚜mol CO_2 m^{-2} s^{-1})	10.10±0.57c	102.60±8.24b	10.20±2.17c	127.95±11.16a
P_n/Shoot DW (µmol CO_2 m^{-2} s^{-1}/g)	0.63±0.05a	0.10±0.01c	0.34±0.02b	0.06±0.02c

Pn, net photosynthesis; gs. stomatal conductance; Ψ_l , leaf water potential; T_l, leaf temperature; RCC, relative chlorophyll content; Shoot DW, shoot dry weight; C, well-watered plants; WS, non-irrigated plants; CM, well-watered and inoculated plants; WSM, non-irrigated and inoculated plants. Different letters in rows indicate significant differences according $Duncan_{0.05}$ test.

4. Discussion

The percentage of mycorrhization of *Pisolithus tinctorius* was not excessively high, despite being an ectomycorrhiza, which has special relevance in this genus with high mycorrhizal capacity (Comandini et al., 2006). In our conditions, the cistus seedlings were cultivated in pots, which prevented the extension of the fungal mycelium and of the ECM roots. Imposed water stress meant a reduction in the percentage of mycorrhization, like plants, fungi are also affected by water limitation (Dell'Amico et al., 2002). Studies of dually colonized plant species such as members of the genus Cistus indicate that ECM may be more sensitive to drought than AMF (Gehring et al., 2006; Querejeta et al., 2009). In fact, in a previous study with AMF (Glomus iranicum var. tenuihypharum) in *Cistus albidus* plants under water stress (Ortuño et al., 2018), the percentage of mycorrhization was 65%, more than double that obtained with *Pisolithus tinctorius*.

Drought is one of the main adverse factors for seasonal plants, especially for plants grown in pots (Sánchez-Blanco et al., 2009; Álvarez et al., 2013; Álvarez et al., 2019). At the end of the experiment, water stress caused a decrease in leaf dry weight, which could be interpreted as a conservative strategy to reduce transpiration and maintain hydraulic conductivity under water depletion (Limousin et al., 2009; Barbeta et al., 2015; Sánchez-Blanco et al., 2002). Despite the low percentage of mycorrhization, ECM improved both shoot and root growth of the plants under well-irrigated conditions. Furthermore, unlike what occurred in Cistus plants inoculated with AMF (Ortuño et al., 2018), *Pisolithus tinctorious* increased root growth under water stress. Usually, more root branching has been found in ECM species than AMF, what is related to higher ability to absorb more water and nutrient, improving plant water relations under low water conditions (Liese et al., 2017; Wu et al., 2017; Liu et al., 2018; Zou et al., 2013; Morte et al., 2009).

Leaf water potential and actual osmotic potential are good indicators of water stress (Álvarez and Sánchez-Blanco, 2013; Álvarez and Sánchez-Blanco, 2015). In our study, plants subjected to water stress showed no osmotic adjustment, perhaps, the speed of the development of water stress and the low inorganic solutes accumulation, such as potassium and sodium, could not contribute to osmo-regulation (Ortuño et al., 2018; Acosta-Motos et al., 2017). The fact that $\Delta\Psi$SS at the end of the experiment in control treatment were almost null indicated that dehydration was the major mechanism involved in $\Delta\Psi$SS, while the more noteworthy $\Delta\Psi$SS in WS treatment seems to indicate that the dehydration could be produced by the transpiration (Sánchez-Blanco et al., 1998). Soil water potential at the root surface (Ψ_r) reflects the accumulation of net solutes allowing reduction of Ψ_l (around −3.0 MPa), in order to guarantee water transport to the leaves (Acosta-Motos et al., 2017; Álvarez et al., 2012; Acosta-Motos et al., 2015; Acosta-Motos et al., 2016). Concerning ECM effect, it improved the plant water status under stress conditions (Augé, 2001). This ameliorative effect has been ascribed to the extended external mycelia of the root systems of ECM fungi, which reach soil pores inaccessible to the roots in water (Smith and Read, 2008; Dixon et al., 1980; Futai et al., 2008).

As a consequence of the reduction of the substrate water potential at the root surface and the leaf water potential in plants subjected to water stress, the stomatal conductance decreased drastically, acting as a mechanism to prevent excessive loss of water (Álvarez et al., 2013; Álvarez et al., 2019; Costa et al., 2012; Costa et al., 2007; Chaves et al., 2016). In our conditions, the relationship between g_s and Ψ_l was adjusted to an exponential curve, so that under water-stress plants exhibited progressively lower Ψ_l, but almost no change in g_s. Likewise, it has been well established that plants regulate rates of transpiration and photosynthesis in parallel, maintaining a balance between g_s and P_n (Lawson et al., 2011). A strong correlation between stomatal conductance with net photosynthesis observed in the current study appears to reflect the gas exchange limitation of photosynthesis (Meng and Arp, 1993; Sánchez-Blanco et al., 2019; Chaves et al., 2009). At the end of the experiment, the imposed water stress induced an increase in intrinsic WUE, which is in agreement with Costa et al. (2007), who reported that deficit irrigation strategies can be successfully applied to different crops, in order to improve water savings. Water deficit by closing stomata, causes increasing leaf temperature, considering the major determinant of leaf temperature is the rate of evaporation or transpiration from the leaf (Jones et al., 2009; Costa et al., 2013; Gómez-Bellot et al., 2015). This behavior was observed at the end of our experiment.

ABA synthesis is one of the fastest responses of plants to abiotic stress causing stomatal closure. In addition, SA is involved in the regulation of drought responses

(Munné-Bosch et al., 2003), inducing the generation of reactive oxygen species (ROS) and, consequently, causing the stomatal closure (Melotto et al., 2006). In our experiment, endogenous ABA and SA levels in stressed plants increased up to of six and almost twofold than those of control plants, respectively, while endogenous 1-aminocyclopropane1-carboxylic acid (ACC) levels in leaves decreased. ACC is the precursor for the production of ethylene and under drought, a close relationship between ACC expression and ethylene synthesis has been demonstrated (Gómez-Cadenas et al., 1996). In these conditions, ACC levels decrease and, therefore, plant growth is reduced. However, some studies suggest that ABA and ethylene are antagonists and have described decreases in ethylene production as a consequence of an increased concentration of ABA in water stressed plants, regulating some drought responses in plants, such as root and leaf growth (Wright, 1980; Tan and Thimann, 1989; Chaves et al., 2003; Olivella et al., 1998; Spollen et al., 2000). This is in accordance with our results, which show that in conjunction with a decrease in stomatal conductance and leaf water potential, ABA in leaves increased, while ethylene decreased, increasing root growth.

Hormonal profiles are also altered to alleviate the negative effects of water stress on mycorrhizated plants (Liu et al., 2018; Fernández-Lizarazo and Moreno-Fonseca, 2016; Miransari et al., 2014; Miransari, 2010). Several studies confirmed that inoculation with mycorrhizal fungi results in decreasing the endogenous ABA and SA (Rincón et al., 2005; Aroca et al., 2013). However, in our assay ECM treatments (CM and WSM) had not significant changes in any of both leaf hormones neither under well-watered nor water stress conditions. Since ABA is known to induce stomatal closure (Zhang et al., 1987), its lower concentration in ECM plants could play a role in the stomatal conductance, allowing no increase in net CO_2 assimilation rate in ECM inoculated plants (Sebastiana et al., 2013). This fact leads no significant improvement in intrinsic water use efficiency (P_n/g_s).

Nevertheless, ECM significantly decreased leaf scopoletin (SC). Scopoletin (6-methoxy7-hydroxycoumarin) is a typical phytoalexin which is an important secondary metabolite synthesized in plants as a defense mechanism against various environmental stresses (Siwinska et al., 2014). Its synthesis is activated once some kind of infection has occurred in plants, but it can also be triggered due to various types of abiotic stresses. Scopoletin has also been found in many other plant species (e.g., Solanaceae, such as tobacco or potato, and sunflower, among others), showing antifungal and antibacterial activity (Siwinska et al., 2014; Tal and Roberson, 1986; Costet et al., 2002). Its accumulation has been correlated with resistance to microbial attack and other stresses, as well as mechanical damage and dehydration (Tanaka et al., 1983). Furthermore, it appears to be the product that most increases in concentration in infected plants compared to other coumarins and coumaric glycosides, such as scopolin, esculetin, and esculin (Uritani, 1999; Buschmann et al., 2000; Giesemann et al., 2008). In our study, SC decreased significantly in mychorrhizal plants indicating that ECM plants have been able to synthesize defense mechanisms against abiotic and biotic stresses against non-mycorrhizal plants, and therefore SC is lower in ECM plants.

In conclusion, the mechanism of C. albidus to avoid water stress was related to its ability to decrease aerial growth and to modify leaf gas exchange, increasing water use efficiency. Water stress positively stimulated the levels ABA and SA, who strongly enhanced drought tolerance. On the other hand, despite the low percentages of mycorrhization, *Pisolithus tinctorious* inoculation in addition to improving plant growth, and slightly gas exchange and quality plant parameters, it improved the water status of Cistus under water stress conditions, probably due to a decrease in SC values, suggesting that the selected ECM can alleviate some of the harmful effects of water scarcity. Therefore, the use of Cistus plants inoculated with *Pisolithus tinctorious* in gardening or restoration projects can be a sustainable economic and environmental option.

CHAPTER 2

Suitability strategies for the use of saline water resources for irrigation in different species

Chapter 2.1

Tolerance and recovery capacity to saline wastewater irrigation of *Salvia officinalis* and *Asteriscus maritimus* plants inoculated with arbuscular mycorrhizae

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Abstract

The use of unconventional water resources is increasingly considered as an alternative in order to mitigate the effects of water scarcity. However, the high levels of salt in treated wastewater can be mitigated by the use of mycorrhizae. In this work attempt to identify which of two species with different levels of salinity tolerance, *Salvia officinalis* L. or *Asteriscus maritimus* L., is more suitable for irrigation with brackish waters, as well as the effect of the arbuscular mycorrhiza *Glomus iranicum* on the plant. The experiment was carried out in a growth chamber with a first phase, during which both inoculated and non-inoculated species were irrigated with good quality water, a second phase in which the plants were irrigated with reclaimed wastewater, and a third phase, in which the plants were irrigated with good quality water again (recovery). The higher Na^+ and Cl^- levels in the substrate did not result in an increase in ions in the aerial part of Asteriscus, and had a less negative effect on growth than in Salvia. Salinity caused a reduction in leaf water potential, stomatal conductance and net photosynthesis in both species. The percentage pf mycorrhization was higher in Asteriscus than in Salvia, mitigating the decrease in leaf water potential due to the presence of salts in the water. There was osmotic adjustment in Salvia, although the proline content increased in both species. The damages produced by the salts were clearer in Salvia, in which lipid peroxidation values were higher. Likewise, the visual appearance of the leaves showed symptoms of toxicity in this species, although the mycorrhizae diminished these effects. Although the Na^+ and Cl^- content of substrate was higher in the recovery phase, irrigation with good quality water induced the recovery of lipid peroxidation in both species, as well as the appearance of new leaves in Salvia.

1. Introduction

Factors such as climatic variations, population growth and ineffective water management practices have caused a great imbalance in the water availability in arid and semi-arid regions (Acosta-Motos, 2014). Taking into account the importance of this resource, especially for crops and plant development, the search of alternative sources to alleviate the water shortage is of vital importance. One of the viable alternatives may be the use of marginal waters, such as reclaimed waters. Technological advances in wastewater treatment in recent years have meant that marginal waters can be considered suitable for agricultural irrigation and for agricultural, industrial or environmental improvement purposes in areas with limited water resources, such as is the case of the Region of Murcia (Grattan et al., 2015). In the last mentioned example, the use of these waters could be considered for the irrigation of ornamental plants for landscaping purposes, in revegetation, urban gardening or leisure spaces. Such is the case of the species under study: *Asteriscus maritimus* and *Salvia officinalis*. Despite the above mentioned advances, these waters are generally characterized by having higher levels of salts than conventional water, accompanied by changes in pH, alkalinity and chemical substances (Gómez-Bellot et al., 2015), which can affect the growth, development and quality of plants.

Numerous investigations have studied the effects of salinity on plants. Excess salts, either in the substrate or in the irrigation water, can affect the development of plants in different ways, since the accumulation of salts lowers the osmotic potential, making it difficult for the plant to absorb water and causing a water deficit. Moreover, there is an ionic effect, a consequence of the presence of toxic ions in plant tissues at concentrations higher than some species can tolerate, which can cause toxicity and nutritional imbalances and lead to oxidative stress. In general, among the effects of salinity on plants we may mention a decrease in photosynthesis and growth, limitations in leaf area, as well as the synthesis of osmoprotective compounds such as proline (Acosta-Motos et al., 2017). The recovery from saline effects through irrigation with good quality water is also important, as has been described in several species, normally this is a situation that occurs naturally and is closely related to the recovery of photosynthesis and capacity of the plants to repair damage to the membranes (Conde et al., 2011; Lorente et al., 2021).

According to their resistance to salinity, plants can basically be classified into two groups (González-Rodríguez, 2016). On the one hand, glycophytes are those species whose growth and development is inhibited by a high concentration of salt, and include species like *Salvia officinalis*. On the other hand, halophytes such as *Asteriscus maritimus* that can easily survive in soils with relatively high concentrations of NaCl (300-500 mM) because they have developed resistance mechanisms (Rodríguez et al., 2005, Acosta-Motos et al., 2017, Gómez-Bellot et al., 2021).

In addition to the physiological mechanisms of such plants, the symbiosis that plants can establish with soil microorganisms and the benefits that this brings has also been studied. One of the most relevant interactions studied is the association between plants and mycorrhizal fungi (Singh et al., 2011), which is estimated to be present in 90% of terrestrial plants (Strasburger, 2002). Specifically, the most common mycorrhizal associations are arbuscular and comprise approximately two thirds of the associations present in terrestrial vascular species (Smith and Read, 2008). Several studies have shown that mycorrhizae can mitigate the negative effects produced by salts, since they increase the water and nutrient absorption capacity of the roots and photosynthesis, and improve the general water status of the plant (Vicente-Sánchez et al., 2014). This effect is greater when the plant is subjected to some type of stress (such as salinity), allowing the survival of the plant under stress conditions (Miransari, 2011; Gómez-Bellot et al., 2015). It, also, has been seen that these fungi play a key role in the proper function of the ecosystems of arid and semi-arid regions of the Mediterranean area (Estrada et al., 2013), genus glomus being the most common (Ruiz Lozano et al., 1995). In the present experiment the arbuscular mycorrhizal fungus *Glomus iranicum* var*. tenuihypharum* was used. This specie is characterized by an extensive mycelium network, improving soil structure and tolerating high amounts of salts, all of which improves the transfer of nutrients from the soil to the plant and the establishment of efficient symbiosis (Błaszkowski et al., 2010). For this reason, it was considered to be of interest to know the role that the fungus plays in the response of two species with different levels of salinity tolerance (*S. officinalis* and *A. maritimus*), when irrigated with reclaimed wastewater. The effects of both variables (irrigation water and mycorrhizae) were evaluated by reference to parameters such as growth, water status and gas exchange, photosynthetic efficiency and lipid peroxidation both in the saline irrigation conditions and when these were replaced by irrigation with good water quality (recovery).

2. Material and methods

2.1. Plant material and experimental conditions

One hundred plants from the nursery were used, fifty *Asteriscus maritimus (L). Less.* and fifty *Salvia officinalis* L., which were transplanted into 1.2 L pots containing a commercial substrate (8:7:1, coconut fibre: Sphagnum peat: perlite). The substrate was supplemented with Osmocote (14:13:13 N, P, K and microelements). After transplantation, the plants were taken to a growth chamber, where they remained until the end of the experiment (four months). The conditions in the chamber were established to promote optimal plant growth: temperature, 23ºC/18ºC (day/night); photosynthetic photon flux density, 350 µmol m^{-2} s^{-1}; photoperiod, 16h/8h (light/dark) and 60% relative humidity (RH).

Picture 1. *Salvia officinalis* (A) and *Asteriscus maritimus* (B) plants in the growth chamber.

2.2. Treatments

At the beginning of the experiment, half of the plants of each species were inoculated with the arbuscular mycorrhizal fungus *Glomus iranicum* var*. tenuihypharum* (15g per 100 mL). During the three weeks after inoculation, all the plants were watered at field capacity to ensure stabilization of the fungus. Then, half of the plants (mycorrhized and non-mycorrhized) of each species were irrigated with reclaimed wastewater (RWW) (170.20 ppm Na^+ and 210.46 ppm Cl^-) with an EC of 3.4-4.0 dS m^{-1} from the wastewater treatment plant (WWTP) located in Roldán-Balsicas (Murcia, Spain). The rest of the plants were irrigated with tap water with an EC of 0.8 dS m^{-1} (36.76 ppm Na^+ and 39.07 ppm Cl^-). As a result, four treatments were obtained per species: plants watered with good quality water, non-mycorrhized (control, C-), and mycorrhized (C+) and plants watered with RWW, non-mycorrhized (RWW-) and mycorrhized (RWW+). The saline period lasted seven weeks. After this period, the saline plants were watered under the same conditions as the control plants for approximately four weeks (recovery period). The experiment was divided into 3 phases based on the irrigation provided:

1. A first “pre-stress” phase, in which all the plants were watered with good quality water (33 days; phase I).
2. A second phase of stress, in which half of the plants were irrigated with RWW (51 days; phase II).
3. A final recovery phase (29 days; phase III), in which the plants were irrigated again with good quality water.

2.3. Fungal colonization

At the end of the experiment, the roots of six plants per treatment were removed from the substrate and washed before staining to evaluate fungal development. The staining process consisted of a first immersion in KOH in a water bath at 100 ºC for 6 minutes, followed by rinsing and a second immersion in H_2O_2. After washing again, it was stained with a trypan blue bath for 15 minutes.

Once stained, a magnifying glass was used to determine the percentage of mycorrhization, following the method described by Kormanik and McGraw (1982): the stained roots were placed in plates for counting and 100 fields were observed, determining the positive fields (colonized) and negative (not colonized). Finally, the percentage of colonization was calculated by the following formula (Sieverding, 1983):

$$\%\ \text{colonization} = \frac{\text{number of colonized fields}}{\text{total number of fields observed}} \times 100$$

2.4. Biomass and leaf area

At the end of both the saline (phase II) and recovery (phase III) phases, five plants per treatment were extracted and separated into roots, stems and leaves. The fresh weight (FW) was then determined and, after drying in an oven at 60 ºC, the dry weight (DW) was obtained.

In order to check whether the higher concentration of salts present in the RWW affected the growth of leaves, leaf area was also studied using a scanner (Area Meter AM 200, ADC BioScientific Ltd, Herts, England) throughout the experiment.

2.5. Substrate and plant mineral content

At the end of the stress and recovery periods, the mineral content of both substrates (5 samples per treatment) and of plants (separated into leaves, stems and roots, 5 plants per treatment) were analysed. For this, the macronutrient concentrations were determined in a digester with HNO3 / HCl4 (2:1, v/v) using an inductively coupled plasma optical emission spectrometer (ICP-OES IRIS INTREPID II XDL, Thermo Fisher Scientific Inc., Loughborough, UK). The concentration of Cl^- in the aqueous extracts obtained was analysed by a chloride analyser (Chloride Analyser Model 926, Sherwood Scientific Ltd.) by mixing 100 mg of powdered dry sample with 40 mL of water before shaking for 30 min and filtering.

2.6. Water relations

Leaf water potential (Ψ_l) was measured periodically at maximum luminosity using a pressure chamber (Soil Moisture Equipment Co., Santa Barbara, CA, USA, mod. 3000) according to the method described by Scholander et al. (1965). The leaves were introduced into the chamber and the pressure was increased at a rate of 0.03 MPa s^{-1} with nitrogen gas, until a drop of sap appeared.

Picture 2. Scholander chamber with a Salvia leaf.

The osmotic potential (Ψ_s) was calculated with a Wescor 5520 vapour pressure osmometer (Wescor Inc., Logan, UT, USA.), calibrated with solutions of known osmolality (Gucci ,1991). The turgor potential (Ψ_p) was calculated as the difference between the leaf and osmotic water potential.

To know the osmotic water potential at full turgor (Ψ_{100s}) the leaves were immersed in distilled water for 24 hours at 4 ºC in the dark. Subsequently, the same procedure described for Ψ_s was followed, taking measurements periodically in five plants per treatment.

2.7. Gas exchange and leaf temperature

Stomatal conductance (g_s) and net photosynthesis rate (P_n) were determined with a portable gas exchange meter, LICOR 6400 (LI-COR Inc., Lincoln, NE, USA), making the measurements at the same time and in the same plants in which the water relations were measured. The measurement parameters were set at a CO_2 concentration of 400 ppm, with an airflow rate of 300 µmol s^{-1} and photosynthetically active radiation (PAR) of 1000 µmol m^{-2} s^{-1}.

Leaf temperature (T_l) was obtained from thermal images taken with a thermographic camera (ThermaCam FLIRe50 System, Inc., UK) periodically during the experiment on five plants per treatment. The background temperature was calculated as the radiation temperature in a wrinkled aluminium paper placed in a position similar to that of the sheets of interest, setting the emissivity value at 1.0 (Jones, 1999). The emissivity for the measurement of the leaves was set at 0.96 (Grant et al., 2007). Images were taken from a distance of 0.5 m and processed with ThermaCam Reacher Professional 2.10 FLIR QuickReport software.

2.8. Chlorophyll fluorescence

Chlorophyll fluorescence measurements were carried out in three leaves per treatment, at the end of each phase, using a fluorimeter (IMAGINGPAM M-series, Heinz Walz, Effeltrich, Germany). First, the leaves were adapted to darkness for 15 minutes to achieve maximum stomata opening. Subsequently, the maximum and minimum fluorescence values were measured. A kinetic analysis was performed in the presence of actinic light (81 µmol quanta m^{-2} s^{-1} PAR) on saturating pulses of light at 27008 µmol quanta m^{-2} s^{-1} PAR every 20 seconds. The photochemical parameters studied were: Fv/Fm (maximum efficiency of PSII), Y (II) (quantum yield) and photochemical quenching (qP). The non-photochemical parameters measured (related to heat dissipation) included non-photochemical quenching (qN).

2.9. Proline content

The proline content was determined in five leaves per plant, at the end of phases I and II, following the method of Bates et al. (1973). For this, the plant material was homogenized with 5 mL of 3% sulfosalicylic acid and centrifuged for 10 minutes at 12000 g. After this, 1 mL of the supernatant was mixed with 1 mL of acidic ninhydrin and 1 mL of glacial acetic acid, and incubated for 1 hour at 100 °C before being rapidly chilled on ice. Extraction was carried out with 2 mL of toluene to achieve full separation of the two phases. The organic phase was extracted from the mixture and its absorbance at 520 nm was measured.

2.10. Lipid peroxidation

Lipid peroxidation is considered a sign of oxidative stress, and so is a parameter of interest to be measured. In this case, the concentration of malondialdehyde (MDA) was measured in five plants per treatment, following the procedure described by Cakmak and Horst (1991), whereby lipid peroxidation is estimated by determining the amount of substances that react with thiobarbituric acid (TBARS). The amount of TBARS was calculated from the difference in absorbance obtained at both wavelengths and a molar extinction coefficient of 155 mM^{-1} cm^{-1}.

2.11. Statistical analysis

In this experiment, 50 plants (per species) were randomly assigned to each treatment. Data were analysed by one-way ANOVA using the statistical analysis program SPSS (IBM SPSS Statistics 26 for Windows, CA, USA). Treatment means were separated with Duncan's Multiple Range test ($P \leq 0.05$). Before statistical analysis, the percentage of root colonization was subjected to an arcsine square root transformation to ensure homogeneity of variance.

3. Results

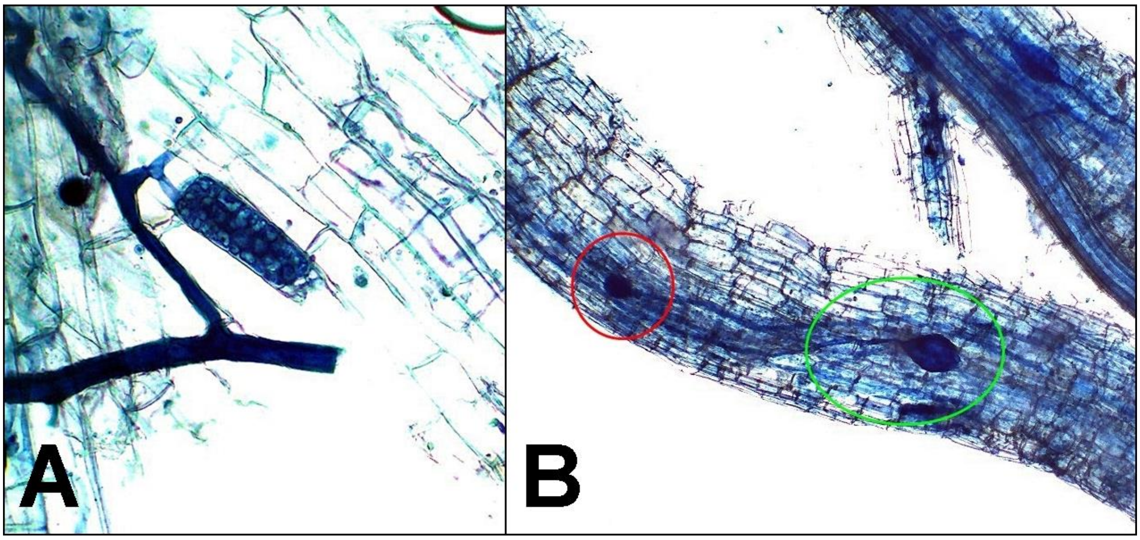
3.1. Mycorrhization percentage

All the inoculated plants were colonized by *Glomus iranicum* var. *tenuihypharum*. However, *Asteriscus maritimus* presented a higher percentage of mycorrhization than *Salvia officinalis*, both in the saline phase (II) and recovery phase (III) (Table 1). Root mycorrhization for both species was higher in phase II than in phase III in the plants irrigated with RWW (RWW+).

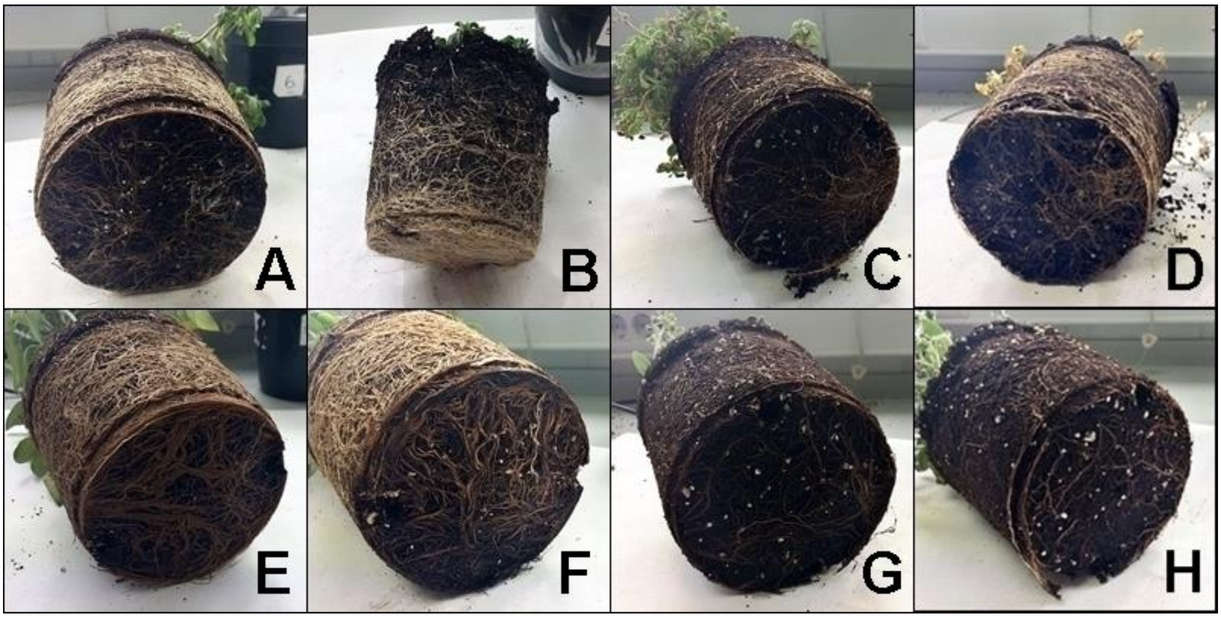
Table 1. Percentage of root mycorrhization on *Asteriscus maritimus* and *Salvia officinalis* plants irrigated with control water (C+) and reclaimed wastewater (RWW+) at the end of the phase II and phase III. Values are means ± SEM (n=6).

	Asteriscus maritimus		***Salvia officinalis***	
	C+	**RWW+**	**C+**	**RWW+**
Phase II	33.00±2.87 a	37.00±4.07 aA	13.70±2.22 b	15.67±2.47 bA
Phase III	29.50±4.65 a	18.00±5.51 bB	10.17±1.93 c	10.33±1.80 cB

Different capital letters indicate significant differences in treatments between phases and lowercase letters in rows indicate significant differences between treatments and species, both according to a Duncan$_{0.05}$ test.



Picture 3. (A) Arbuscle in *Asteriscus maritimus* root. (B) Spore (red) and vesicle (green) in the root of *Salvia officinalis*.



Picture 4. Root development according to treatments. *Asteriscus maritimus* (A-D): C- (A), C+ (B), RWW- (C), RWW+ (D). *Salvia officinalis* (E-H): C- (E), C+ (F), RWW- (G), RWW+ (H).

3.2. Biomass and leaf area

Plant growth in Salvia was more affected by irrigation with RWW than in Asteriscus, which presented similar values of leaf and stem dry weight, regardless of the treatments and the phases (Figure 1a and c). However, the root dry weight of the plants irrigated with

RWW (RWW-) was lower than that observed in the control (C-) plants in phase III (Figure 1e).

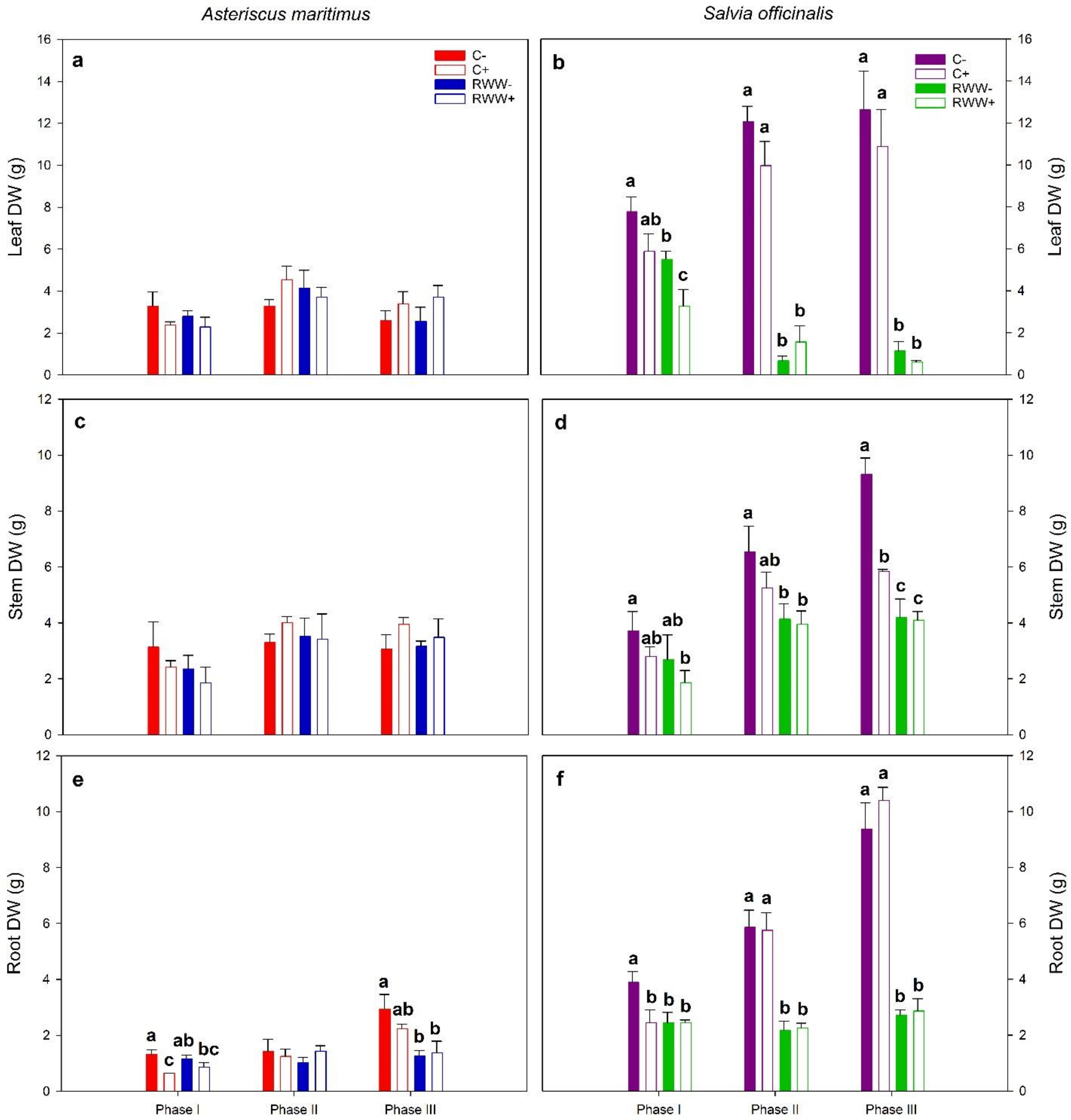
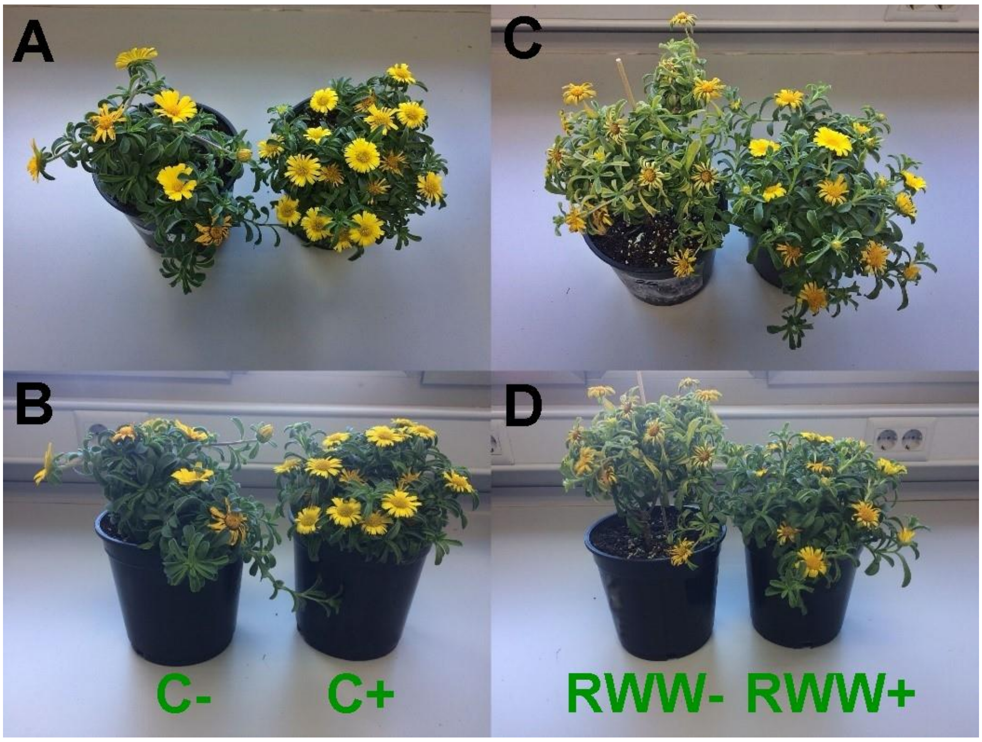


Figure 1. Dry weight (DW) of leaf, stem and root of *Asteriscus maritimus* (a,c,e) and *Salvia officinalis* (b,d,f) subjected to control water and reclaimed wastewater without and with arbuscular mycorrhizal fungi at the end of the three phases of the experiment. Values are means of five samples. The vertical bars indicate standard errors. Different lowercase letters indicate significant differences between treatments according to a Duncan$_{0.05}$ test, and the absence of the same means that there were no such differences.

Salvia behaved differently and, from phase II onward, leaf and root biomass of Salvia plants irrigated with RWW were significantly lower than in control plants. The total biomass (leaf, stem and root) of the control plants in phase II was around 22.525 g, while for plants irrigated with RWW was 7.750 g (Figure 1b, d and f). Furthermore, this reduction in total DW was maintained in the phase III, while control plants had a growth increase through the experiment.

No differences in total DW between treatments and species due to the effect of mycorrhizae were observed (Figure 1).



Picture 5. Aerial (A and C) and front (B and D) view of treatments of *Asteriscus maritimus* at the end of phase II

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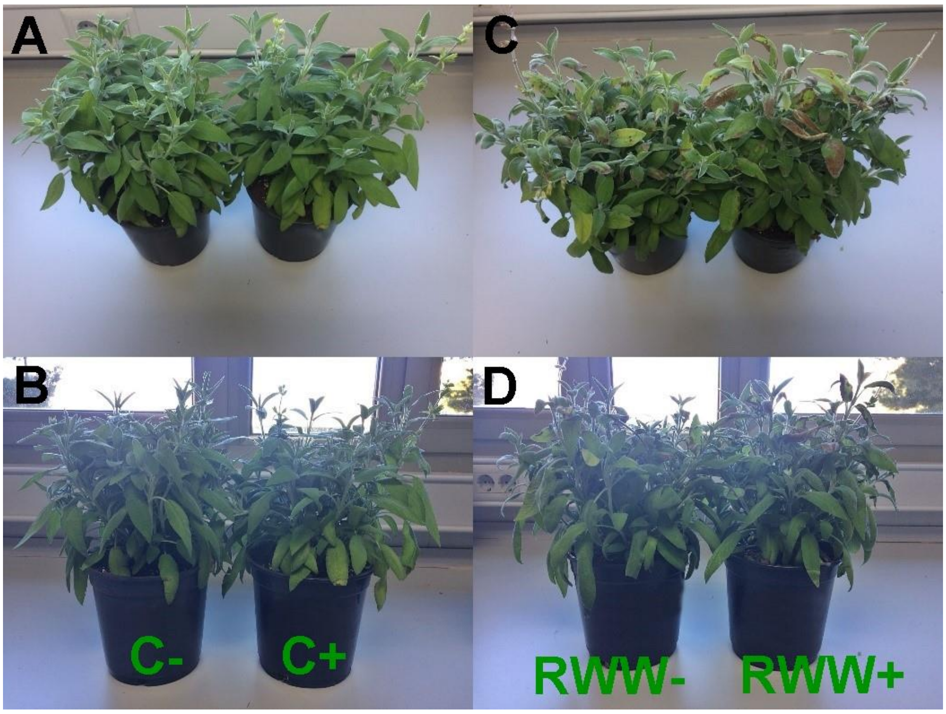


Picture 6. Aerial (A and C) and front (B and D) view of treatments of *Salvia officinalis* at the end of phase II.

Table 2. Leaf area of *Asteriscus maritimus* and *Salvia officinalis* irrigated with control water and reclaimed wastewater, with and without arbuscular mycorrhiza in the three phases of the experiment. Values are means ± SEM (n = 5).

		Leaf area (mm^2)	
		Asteriscus maritimus	***Salvia officinalis***
Phase I	**C-**	257.25±12.75 a	915.92±8.03 a
	C+	177.21±4.04 b	931.42±7.58 a
	RWW-	177.33±6.67 b	821.17±8.62 b
	RWW+	169.72±2.26 b	938.75±8.62 a
Phase II	**C-**	170.25±4.58	1064±31 a
	C+	146.04±15.79	906.92±37.92 b
	RWW-	180.50±3.83	658.75±13.58 c
	RWW+	166.92±14.42	718.00±21.17 c
Phase III	**C-**	194.67±12.93 a	533.33±7.37 a
	C+	197.83±0.00 b	528.17±12.11 a
	RWW-	171.33±9.25 b	318.17±29.67 b
	RWW+	158.25±13.41 b	306.67±23.81 b

Different letters in columns indicate significant differences between treatments in each phase according to a Duncan$_{0.05}$ test. Absence of letters in rows indicates no significant differences between treatments.

At the beginning of the experiment, Asteriscus plants irrigated with control water (C-) showed higher values of leaf area. However, during phase II, there were no significant differences between treatments. At the end of the phase III, leaf area in the control plants (C- and C+) was higher than in plants irrigated with RWW (RWW- and RWW+) (Table 2).

In Salvia, the decrease in leaf area was more evident in phase II, when the plants were irrigated with RWW, showing a significantly smaller area than the controls. These differences were maintained in phase III (Table 2).

3.3. Substrate and plant mineral content

3.3.1. Na^+ and Cl^- ion content in the substrate

Na^+ and Cl^- contents of the substrates of the saline treatments were significantly higher than those of the control for both species and in both phases (phase II and phase III) (Table 3). In Asteriscus in phase II, the mycorrhizae mitigated the accumulation of Na^+ in the substrates of the plants irrigated with RWW (RWW+) compared with the non-mycorrhizal plants (RWW-). In Salvia, the highest Cl^- content was observed in the substrates of RWW+ plants at the end of the recovery phase (Phase III) (Table 3).

Table 3. Substrate Na^+ and Cl^- content of *Asteriscus maritimus* and *Salvia officinalis* irrigated with control water and reclaimed wastewater, with and without arbuscular mycorrhiza at the end of the phase II and phase III. Values are means ± SEM (n =5).

		Asteriscus maritimus		***Salvia officinalis***	
		Na^+ (mg/L)	**Cl^- (mg/L)**	**Na^+ (mg/L)**	**Cl^- (mg/L)**
Phase II	**C-**	6.48±0.45 b	4.90±0.31 b	5.76±1.86 b	6.62±0.34 b
	C+	7.03±0.14 b	4.33±0.55 b	6.85±0.35 b	6.67±1.58 b
	RWW-	13.64±0.5 a	13.91±1.35 a	16.04±3.38 a	26.43±2.09 a
	RWW+	10.50±2.85 ab	9.84±1.14 a	16.99±2.97 a	28.22±3.55 a
Phase III	**C-**	7.55±0.25 b	5.31±2.88 b	8.02±1.24 b	9.65±2.51 c
	C+	8.56±1.45 b	6.87±0.27 b	7.98±1.24 b	9.84±2.59 c
	RWW-	19.46±1.33 a	23.99±5.85 a	19.12±0.56 a	27.56±1.30 b
	RWW+	20.70±1.51 a	21.86±3.69 a	21.24±0.98 a	33.65±2.56 a

Different letters in rows indicate significant differences between treatments according to a Duncan $_{0.05}$ test.

3.3.2. Na^+ and Cl^- ions content in root and aerial part

In general, Salvia plants had a lower Na^+ and Cl^- content than Asteriscus, which showed no significant differences in this respect whether irrigated with RWW or good quality water at the end of the phase II (Table 4). This behaviour remained similar in the recovery phase (phase III), except that mycorrhizae increased the concentration of Cl^- in in the aerial part of plants irrigated with RWW (Table 4A). In Salvia, the Na^+ and Cl^- content was higher in the aerial part of the plants irrigated with RWW than in control plants in phase II (Table 4B). This difference was more pronounced in the recovery phase (phase III), reaching values of approximately 17 mg L^{-1} Na^+ and 88 mg L^{-1} Cl^- in RWW plants compared with the 1.8 mg L^{-1} Na^+ and 13.8 mg L^{-1} Cl^- in control plants. Mycorrhizae tended to decrease the Na^+ and Cl^- content in the RWW+ plants with respect to the RWW- plants in phase III (Table 4B).

Table 4. Aerial and root Na$^+$ and Cl$^-$ content of *Asteriscus maritimus* (A) and *Salvia officinalis* (B) irrigated with control water and reclaimed wastewater, with and without arbuscular mycorrhiza at the end of the phase II and phase III. Values are means ± SEM (n =5).

A

		Asteriscus maritimus			
		Na$^+$ (mg/L)		**Cl$^-$ (mg/L)**	
		Aerial Part	**Roots**	**Aerial Part**	**Roots**
Phase II	**C-**	59.71±4.62	12.39±4.53	88.68±30.30	8.72±2.65
	C+	60.65±3.69	8.57±0.56	93.77±31.73	5.65±0.88
	RWW-	66.59±12.22	14.21±1.12	107.08±32.59	10.08±4.19
	RWW+	68.81±4.57	13.74±4.31	100.26±36.51	12.53±3.24
Phase III	**C-**	23.80±1.58	9.04±3.27	191.82±14.27b	8.08±1.84
	C+	59.90±9.39	15.92±11.97	224.36±17.56ab	6.79±1.77
	RWW-	23.25±1.90	11.28±1.05	165.08±70.78b	6.99±3.13
	RWW+	50.15±25.07	10.27±3.79	300.67±17.56a	11.88±4.54

B

		Salvia officinalis			
		Na$^+$ (mg/L)		**Cl$^-$ (mg/L)**	
		Aerial Part	**Roots**	**Aerial Part**	**Roots**
Phase II	**C-**	1.80±0.20b	4.84±0.49	10.42±1.47b	8.169±0.87a
	C+	2.57±0.19ab	4.51±0.13	9.27±1.15b	6.295±0.16b
	RWW-	4.71±1.69a	4.97±0.81	20.03±1.94a	7.863±3.09ab
	RWW+	3.91±0.88ab	5.28±0.85	20.59±2.78a	9.120±1.70a
Phase III	**C-**	2.05±0.12b	4.74±1.03	12.63±3.27b	10.67±2.00
	C+	1.57±0.34b	5.29±0.43	14.89±2.77b	9.68±1.35
	RWW-	18.25±9.34a	9.07±4.66	104.76±45.03a	12.04±9.96
	RWW+	15.82±3.02a	7.51±1.43	72.66±10.25a	9.84±1.14

Different letters in columns indicate significant differences between treatments according to a Duncan$_{0.05}$ test. Absence of letters in columns indicates no significant differences between treatments.

3.4. Water relations

The leaf water potential (Ψ_l) in *Asteriscus maritimus* decreased in plants irrigated with RWW during phase II. Mycorrhization alleviated the negative effect of water stress due to salts and the decrease in Ψ_l values was less pronounced (Figure 2a). When the plants were irrigated with good quality water (phase III), Ψ_l values recovered (Figure 2a). No differences were observed between treatments at any time during the experiment in the case of osmotic water potential (Ψ_s) (Figures 2c), although turgor potential (Ψ_p) showed differences in both phase II and phase III. In phase II, RWW+ plants presented higher Ψ_p values than the non-mycorrhized (RWW-) and in phase III the value of Ψ_p of RWW+ were similar to C+ (Figure 2g).

Differences between treatments in water relations were clearer in *Salvia officinalis*. The Ψ_l, Ψ_s, and Ψ_{100s} values of the salinized plants were significantly more negative than in the control during phase II (Figure 2b, d, f). Likewise, the Ψ_p in the saline plants was higher than the control plants (Figure 2h). Mycorrhizae had no effect on these parameters. In phase III, Ψ_{100s} decreased when the plants were watered with RWW (Figure 2f) and mycorrhization had no effect on Ψ_{100s}. In this phase, the most negative values of Ψ_{100s} were observed in the RWW+ plants (Figure 2f).

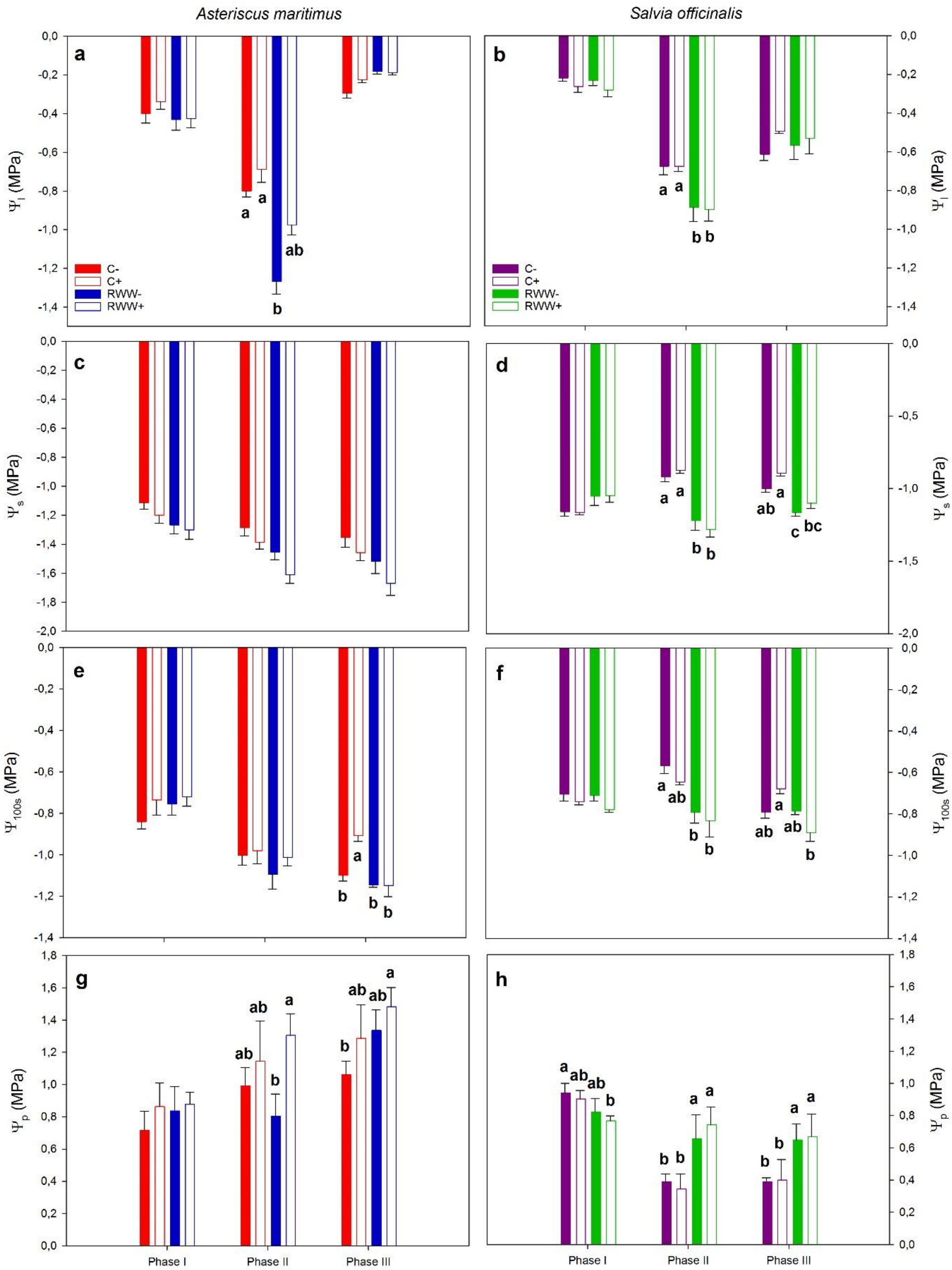


Figure 2. Leaf water potential (Ψ_l), osmotic water potential (Ψ_s), osmotic water potential at full turgor (Ψ_{100s}) and turgor potential (Ψ_p) of *A. maritimus* (a.c.e,g) and *S. officinalis* (b,d,f,h) irrigated with control water and reclaimed wastewater, without and with arbuscular mycorrhizal fungi in the three phases of the experiment. Values are means of five plants. The vertical bars indicate standard errors. Different lowercase letters indicate significant differences between treatments according to a Duncan$_{0.05}$ test and their absence that there are no such differences.

3.5. Proline content

The plants of both species irrigated with RWW had higher proline content than the controls in phase II (Figure 3). Furthermore, while the effect of mycorrhization was not observed in Salvia, mycorrhized Asteriscus plants had a slightly higher proline concentration than non-mycorrhized plants (Figure 3).

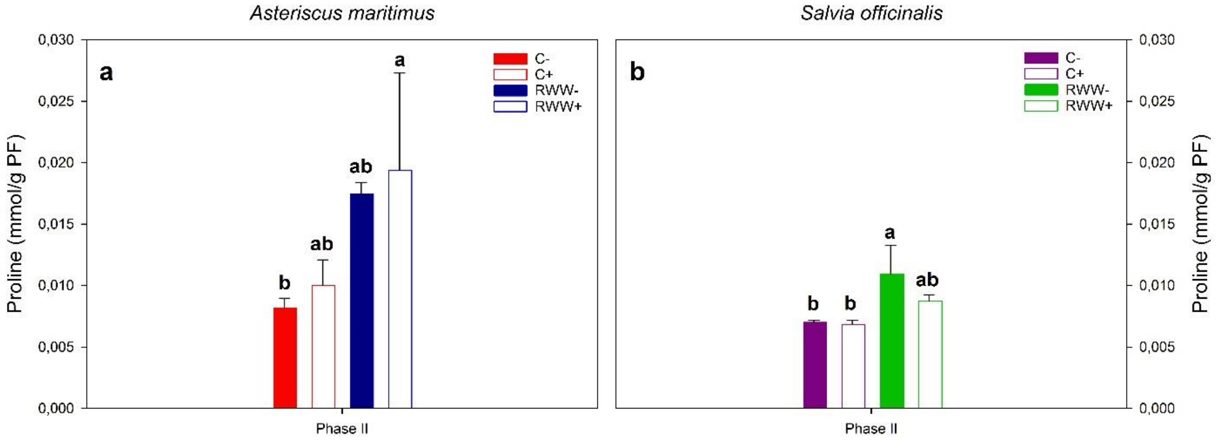


Figure 3. Proline content of *Asteriscus maritimus* (a) and *Salvia officinalis* (b) subjected to control water and reclaimed wastewater without and with arbuscular mycorrhizal fungi during phase II. Values are means of five plants. The vertical bars indicate standard errors. Different lowercase letters indicate significant differences between treatments according to a Duncan$_{0.05}$ test.

3.6. Gas exchange and thermography

In Asteriscus, stomatal conductance (g_s) was lower during phase II in RWW- and RWW+ plants (Figure 4a). This caused an increase in leaf temperature (foliar heating). However, during phase III, the g_s values of RWW- and RWW+ plants recovered to reach the levels of control plants or were even higher in RWW+. Thus, this increase in g_s was accompanied by a decrease in T_l (Figure 4a and c).

Similar behaviour was observed in Salvia in phase II, when g_s decreased in saline plants (RWW- and RWW+). The highest g_s values were found in C+ plants. At the end of phase III, no differences were observed between treatments. Again, the decrease in g_s coincided with increases in T_l (Figure 4b, d).

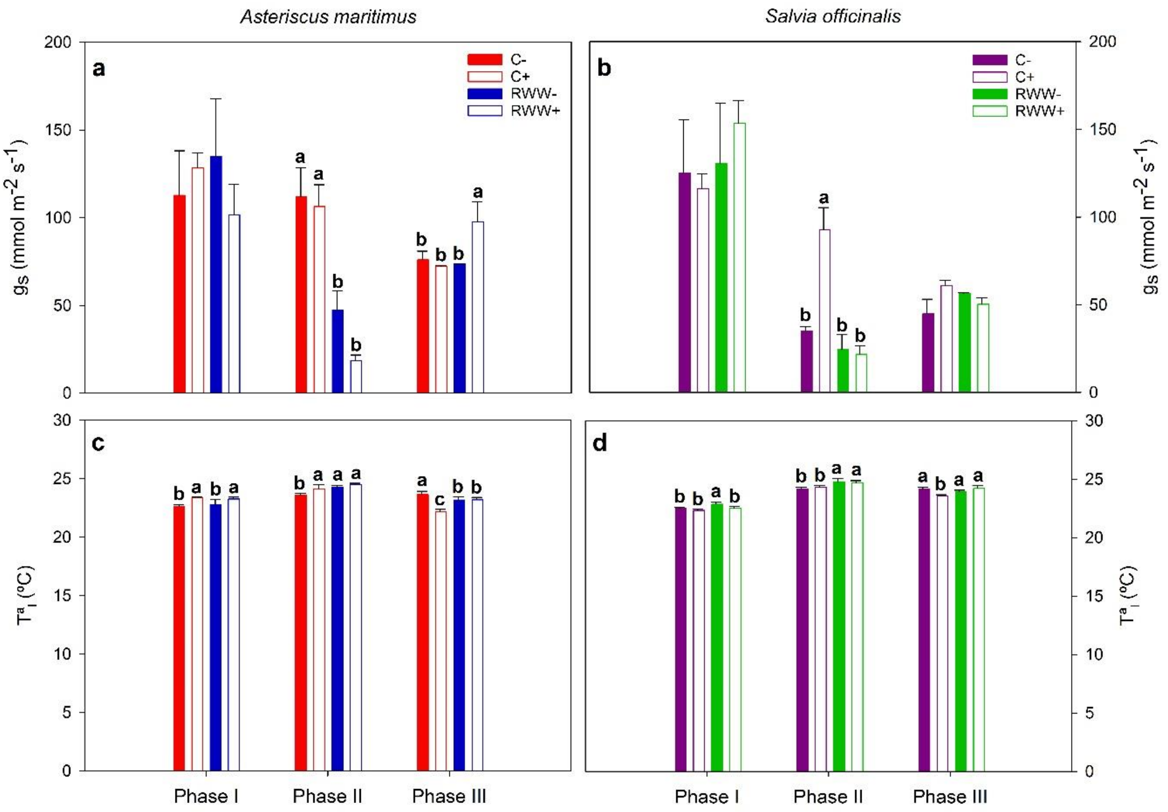


Figure 4. Stomatal conductance (g_s) and leaf temperature (T_l) of *Asteriscus maritimus* (a,c) and *Salvia officinalis* (b,d) subjected to control water and reclaimed wastewater without and with arbuscular mycorrhizal fungi in the three phases of the experiment. Values are means of five plants. The vertical bars indicate the standard error. Different lowercase letters indicate significant differences between treatments according to a $Duncan_{0.05}$ test and their absence that there are no such differences.

Net photosynthesis (P_n) was affected by saline water in both species (Figure 5). In Asteriscus, the saline treatments (RWW- and RWW+) led to lower P_n values in phase II. Furthermore, the mycorrhized plants (RWW+) had significantly lower P_n values than the non-mycorrhized plants. At the end of phase III, P_n values had recovered and were higher than in the control plants (Figure 5a).

Similar P_n behaviour was observed in Salvia plants in phase II, when the control plants had significantly higher P_n than those irrigated with RWW. Mycorrhization significantly increased P_n values in the control plants. After the RWW- and RWW+ plants had recovered (phase III), P_n values were the same as in the control plants (Figure 5b).

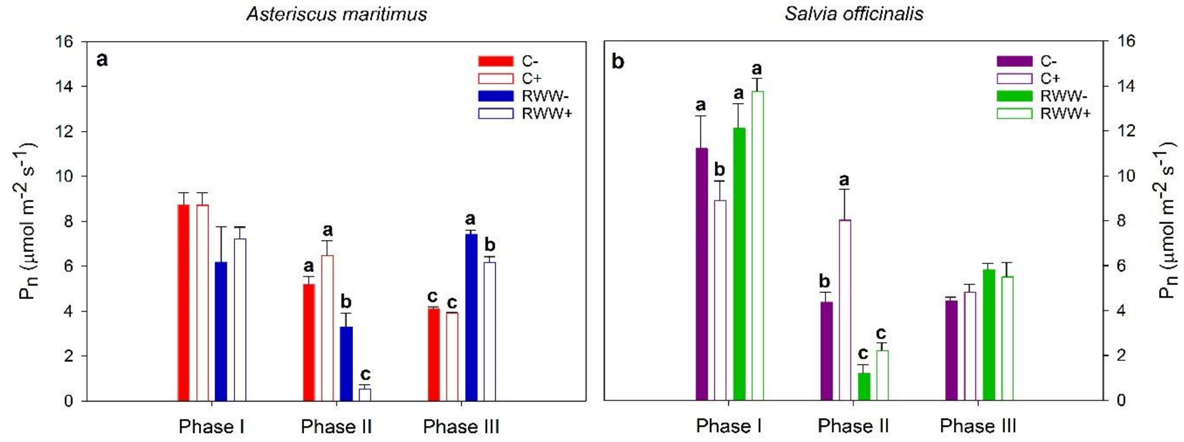


Figure 5. Photosynthetic rate (P_n) of *Asteriscus maritimus* (a) and *Salvia officinalis* (b) subjected to control water and reclaimed wastewater without and with arbuscular mycorrhizal fungi in the three phases of the experiment. Values are means of five plants. The vertical bars indicate standard error. Different lowercase letters indicate significant differences between treatments according to a $Duncan_{0.05}$ test and their absence that there are no such differences.

3.7. Chlorophyll fluorescence

No differences in Fv/Fm were observed in *Asteriscus maritimus* as a result of the type of water or mycorrhizae in phase II. However, RWW- treatment presented the lowest values of Y(II) and qP, while RWW+ had similar to control plants (C- and C+) (Figure 6C). In the recovery phase (phase III), the quantum yield (Y(II)) of the RWW- plants was similar to that of the other treatments, while the photochemical quenching (qP) continued to decrease in the RWW- and RWW+ plants (Figure 6E). In the case of the non-photochemical parameters (qN) a similar trend was observed, C- and C+ plants also had higher values than RWW- and RWW+ in phase II (Figure 6C).

Both the photochemical and non-photochemical parameters were negatively affected by the RWW in *Salvia officinalis* (Figure 6D, F). The Fv/Fm was affected in phase II, when there were significant differences between control plants and those irrigated with RWW. Furthermore, qP and Y(II) noticeably decreased in RWW+ and RWW-, the values of Y(II) of the saline treatment plants being around 30% lower than those of control plants (Figure 6D). However, the decrease in photochemical performance was lower in mycorrhized plants, and their recovery was better (Figure 6D, F).

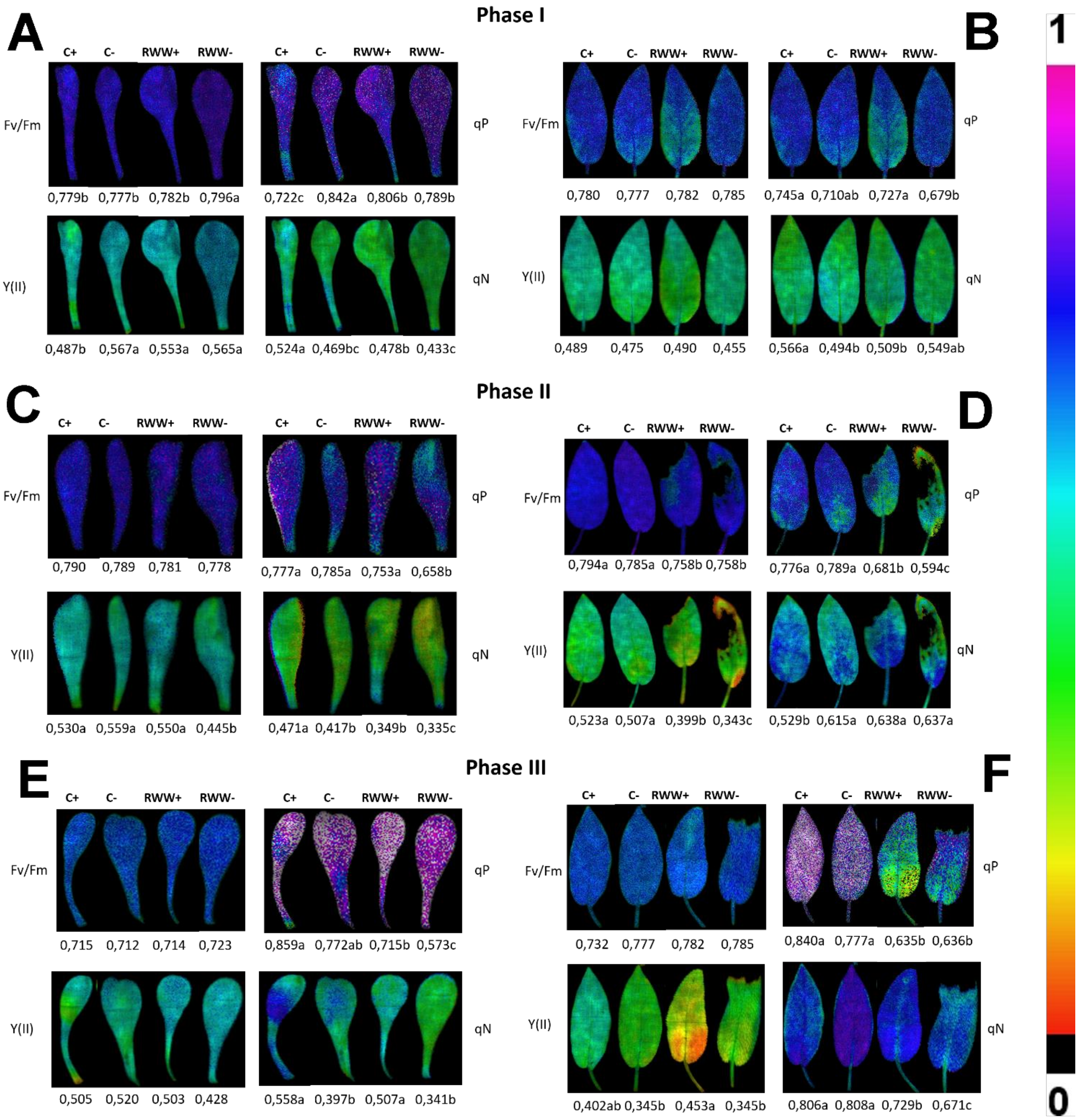
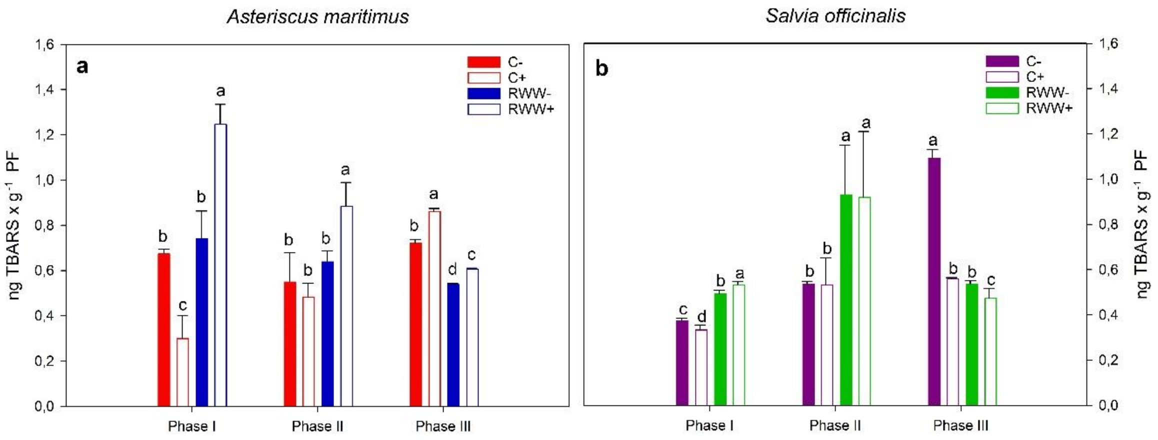


Figure 6. Leaf chlorophyll fluorescence (Fv/Fm, efficiency of PSII; Y(II), quantum yield; qP, photochemical quenching; and qN, non-photochemical quenching) of *Asteriscus maritimus* (A,C,E) and *Salvia officinalis* (B,D,F) watered with control water and reclaimed wastewater without and with arbuscular mycorrhizal fungi at the end the three experimental phases. Values are means of three plants. The vertical bars indicate standard error. Different lowercase letters indicate significant differences between treatments according to a Duncan 0.05 test and their absence that there are no such differences.

3.8. Lipid peroxidation

Regarding the lipid peroxidation of *A. maritimus*, there were significant differences from phase I. In phase II, the peroxidation levels of the different treatments were similar, except those of mycorrhized plants irrigated with RWW (RWW+) that showed significantly higher peroxidation values. In phase III, the peroxidation of the plants of the RWW- and RWW+ treatments decreased below the values of the control plants (Figure 8). The values of the mycorrhized plants were higher than those of the non-mycorrhized ones.

In the case of Salvia plants, the effect of salt was more evident, since differences between plants irrigated with control water and those irrigated with RWW were observed in phase II. In phase III, peroxidation levels decreased in salinized plants, when they were equal to C+ values, while C- plants reached the highest values (Figure 7).



Asteriscus maritimus

Salvia officinalis

Figure 7. Lipid peroxidation of *Asteriscus maritimus* (a) and *Salvia officinalis* (b) subjected to control water and reclaimed wastewater without and with arbuscular mycorrhizal fungi in the three phases of the experiment. Values are means of five plants. The vertical bars indicate standard error. Different lowercase letters indicate significant differences between treatments according to a Duncan$_{0.05}$ test and their absence that there are no such differences.

4. Discussion

Plants are exposed to a variety of abiotic stresses, one of the most problematic being saline stress, which affects growth and yield and can cause plant death (Aggarwal et al., 2012). Because of the shortage of water in Mediterranean areas, the use of unconventional water, such as reclaimed water, for irrigation purposes is increasing (Pedrero et al., 2020). One of the main problems of these waters is their high content of salts, which, in the long term, can lead to salinization of the soil, representing a risk to production (Hameed et al., 2014). Among the lines of research to mitigate the negative effect of salts on crops, are those looking at the use of biological resources, such as the application of mycorrhizae. The use of arbuscular mycorrhizae contributes to improving resistance to salinity by increasing the availability of water and nutrients (Singh et al., 2011).

Both species under study were colonized by the fungus *Glomus iranicum* var*. tenuihypharum*, although the mycorrhization percentage was higher in *Asteriscus maritimus* than in *Salvia officinalis*, since the mycorrhizal dependence of this species is known to be greater (Estrada et al., 2013). However, in our case, the percentage of glomus mycorrhization in Asteriscus was lower than in other studies (Estrada et al., 2013), which may explain why its effect was not appreciated in many of the parameters studied. It must be taken into account that the experimental conditions (limitation of root system development) and salinity affect the development of the fungus, reducing the formation of mycelium and making it difficult to colonize plants (Abdel-Fattah et al., 2016; Kumar et al., 2010).

In both species, the percentage of mycorrhization was higher in the saline phase than in the recovery phase, since the levels of Na^+ and Cl^- in the substrate were higher in this last phase, probably because the salts were not completely washed off with irrigation. Regarding the content of salts in the substrate, *A. maritimus* had higher Na^+ and Cl^- levels in the plants irrigated with RWW than the controls, although this did not increase ions in the aerial part of the plant. Plants try to prevent toxic ions from reaching the aerial part through mechanisms such as salt exclusion or compartmentalization of the same in vacuoles. Halophytes usually have both mechanisms, which allow them to tolerate high concentrations of salts (Acosta-Motos et al., 2017, Gómez-Bellot et al., 2021). However, glycophytes have a low capacity for exclusion and compartmentalization (Gómez-Bellot et al., 2021). We observed that the amount of Na^+ and Cl^- was higher in the Asteriscus plants than in Salvia, but there were no significant differences between the control and saline plants. While Asteriscus was able to exclude salts, Salvia was not, so the plants watered with RWW had higher amount of salts than the control plants. As a result, Asteriscus presented fewer negative effects than Salvia, as seen from the growth results and physiological parameters.

One of the main consequences of excess salts is the reduction in plant growth (Acosta-Motos et al., 2017; Acosta-Motos et al., 2014; Gómez-Bellot et al., 2013). Numerous studies show that the dry weight of tissues subjected to saline stress is lower than in control plants (Acosta-Motos et al., 2014; Valdez-Aguilar et al., 2011), although this negative effect is usually less visible in mycorrhized plants, since they grow better in saline conditions than non-mycorrhized plants. Our results confirmed that the salinized plants had lower growth and it was evident in Salvia. Under these conditions, no effect of mycorrhization was observed on this parameter. However, it should be noted that Kumar et al. (2010) found that even mycorrhized plants may show a significant decrease in dry weight.

Another typical response to salt is a reduction in leaf area, as observed in Salvia plants. This can be considered as an avoidance mechanism, since it minimizes water loss through transpiration (Ruiz-Sánchez et al., 2000, Álvarez et al., 2018). Together with stomatal closure, it favours the retention of ions in the roots or in the soil, hindering their absorption and limiting their accumulation in the aerial part (Koyro, 2006). The reduction of leaf area in Salvia was evident both during phase II and phase III, meaning that that biomass synthesis did not recover during this last phase. No reduction in leaf area was observed in Asteriscus during phase II, but was observed in phase III, when the leaf area of saline plants was lower than that of control plants. Regarding water relations, the accumulation of salts in the rhizosphere causes a decrease in osmotic potential and, therefore, in water potential, reducing the amount of water available for the roots. The first effects of saline stress are practically the same as those of water stress (Acosta-Motos et al., 2017). This was seen clearly, since in *A. maritimus*, and especially in *S. officinalis*, the plants subjected to salinity presented more negative leaf potentials than the controls. Mycorrhizae contribute to maintaining the water status of the plant under stress conditions (Kumar et al., 2010; Hameed et al., 2014) by promoting development and increasing the hydraulic conductivity of the roots (Vicente-Sánchez et al., 2014). This was evident from the leaf water potential of Asteriscus, since the mycorrhized plants irrigated with RWW (RWW+) suffered a lower decrease in potential than the non-mycorrhized ones.

Regarding the behaviour of the osmotic potential at full turgor (Ψ_{100s}), Asteriscus showed no difference between treatments, while in Salvia it decreased in the plants irrigated with RWW (both RWW- and RWW+). An advantage of studying the osmotic water potential at full turgor is that, if the dehydration factor is overlooked, the parameter indicates if there has been osmotic adjustment. Proline stands out among the compounds that can act as osmolytes, increasing under stress conditions (Abdel-Fattah et al., 2016). Proline has antioxidant properties and acts by providing resistance to plants against environmental stresses (Acosta-Motos et al., 2017). In both species, during phase II, proline production increased, which, in Salvia, corresponded to a lower osmotic water potential at full turgor. Even so, the proline levels in both cases were not much higher than the control. In Salvia, a slight increase in proline (in plants irrigated with regenerated water) allowed the turgor potential (Ψ_p) to remain stable and even to reach higher levels than in the control plants. No effect of mycorrhization was observed in Salvia, but in Asteriscus, mycorrhizal plants (RWW+) presented a higher content than non-mycorrhizal plants, despite the fact that several experiments have suggested that fungi promote the accumulation of proline (Evelin et al., 2009; Abdel-Fattah et al., 2016).

When plants are subjected to osmotic stress caused by salinity, one of the first responses is stomatal closure to reduce the water loss through transpiration. Therefore, salinity also causes a decrease in stomatal conductance (Álvarez et al., 2018). In this case, both in Asteriscus and Salvia, the lowest stomatal conductance values coincided with the salinization phase. At the same time, a decrease in stomatal conductance has several effects, such as increasing leaf temperature. In the plants irrigated with regenerated water, there was a significant increase in leaf temperature, which coincided with the lowest values of stomatal conductance. Although some studies have indicated that mycorrhizae promote greater stomatal opening and, therefore, lower leaf temperature (Gómez-Bellot et al., 2015), this was not reflected in our study.

Stomatal closure is one of the factors by which the photosynthetic rate decreases under salinity. In both *A. maritimus* and *S. officinalis*, the photosynthetic rate was affected during irrigation with RWW. Mycorrhized plants have been shown to maintain a higher photosynthetic rate than non-mycorrhized plants under stress conditions (Kumar et al., 2010; Borde et al., 2010). Although in Salvia the mycorrhizae had no effect during phase II, in Asteriscus the opposite was true, and it was the non-mycorrhizal plants (RWW-) that presented a higher photosynthetic rate than inoculated plants.

Photosynthetic activity can also be measured by reference to chlorophyll fluorescence parameters. In general, under salinity conditions, photochemical parameters decrease and non-photochemical parameters increase, since they constitute a mechanism to dissipate excess energy. In our experiment, the values of the photochemical parameters decreased in both species during the stress period, but the non-photochemical parameters did not increase (although it is true that such changes depend to a great extent on the species and the degree of stress). For example, Moradi & Ismail (2007) found that in some cases non-photosynthetic parameters also suffer a decrease as a consequence of stress. In this case, it seems that mycorrhizae have a positive effect, since the photochemical parameters of both species are significantly higher in mycorrhizal plants (RWW+) than in non-mycorrhizae (RWW-), as numerous investigations have previously shown (Abdel-Fattah et al., 2016; Zuccarini et al., 2008; Sheng et al., 2008).

If salt stress is prolonged, Na^+ and Cl^- ions can accumulate inside the cell and lead to oxidative stress. One of the consequences of this stress is the production of reactive oxygen species (ROS), which, if they interact with membrane lipids, can lead to their degradation in a process known as lipid peroxidation (Acosta-Motos et al., 2014). The results were clearer in Salvia, where salinized plants and control plants differed in phase II, the values in saline plants being higher, as would be expected when subject to stress. Although mycorrhizal plants generally tend to have lower peroxidation levels (Abdel-Fattah et al., 2016), in our case there was no apparent effect of mycorrhizae. After recovery, we observed how, in both species, the peroxidation of plants treated with RWW fell to reach values lower than the control values. This could be because, as the plants age, peroxidation increases. During phase III, the plants (especially *S. officinalis*) grew new leaves, and so the number of new and healthy leaves was higher. These young leaves could explain why the plants treated with salt showed less peroxidation than the control plants (older leaves).

To sum up, it was confirmed that the Asteriscus is more tolerant to salt than Salvia, since all the parameters studied presented better values. Irrigation with RWW decreases the ornamental quality of Salvia, making it difficult to use in landscaping projects (Aslani and Razmjoo, 2018). The effect of mycorrhizae was only appreciated in some of the parameters, but did not represent any significant improvement in most cases.

In conclusion, both species are susceptible to mycorrhization with *Glomus iranicum* var. *tenuihypharum*, although the crop conditions limited the mycorrhization percentages. All the measured parameters were less affected in *A. maritimus* than in *S. officinalis*, confirming that it is more tolerant to salinity, which is why it is more suitable for irrigation with reclaimed waters. This tolerance of *A. maritimus* is due to the reduction in the ability of potentially toxic ions to reach the aerial part. *Glomus iranicum* var. *tenuihypharum* improved the photochemical parameters of both species during phase II, as well as the leaf water potential and proline content in *A. maritimus*. And finally, irrigation with good quality water after a period of irrigation with RWW induces the recovery of gas exchange, water status and the ability of plants to repair membranes damage.

Chapter 2.2

Recycled Wastewater and Reverse Osmosis Brine use for halophytes irrigation: differences in physiological, nutritional and hormonal responses of *Crithmum maritimum* and *Atriplex halimus* plants

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Abstract

Halophytes are capable of coping with excessive NaCl in their tissues, although some species may differ in their degree of salt tolerance. In addition, it is not clear whether they can tolerate other confounding factors and impurities associated with non-conventional waters. The experiment was performed in a greenhouse with *Crithmum maritimum* and *Atriplex halimus* plants, growing on soil and irrigated with two different water types: reclaimed wastewater (RWW) (EC: 0.8–1.2 dS m^{-1}) and reverse osmosis brine (ROB) (EC: 4.7–7.9 dS m^{-1}). Both species showed different physiological and nutritional responses, when they were irrigated with ROB. *Atriplex* plants reduced leaf water potential and maintained leaf turgor as consequence of an osmotic adjustment process. *Atriplex* showed higher intrinsic water use efficiency than *Crithmum*, regardless of the type of water used. In *Crithmum*, the water status and photosynthetic efficiency were similar in both treatments. *Crithmum* presented a higher leaf accumulation of B and Ca ions, while *Atriplex* a higher amount of K, Mg, Na and Zn. *Crithmum* plants irrigated with ROB presented higher concentrations of 1-aminocyclopropane-1-carboxylic acid and trans-zeatin-glucoside, whereas abscisic acid concentration was lower. *Atriplex* showed a lower concentration of trans-zeatin-riboside and scopoletin. The characteristics associated to water irrigation did not influence negatively the development of any of these species, which confirms the use of brine as an alternative to irrigate them with conventional waters.

Keywords: salinity; non-conventional irrigation; water status; photosynthetic efficiency; plant nutrition; phytohormones; growth.

1. Introduction

The Mediterranean region is characterized by a climate with prolonged drought periods where isolated and torrential rains are frequent. In addition, soils usually present some extent of salinization, as well as a poor structure and a scarce vegetation cover, which increases water erosion (Benet and Castilla, 2005). Aridity might increase in this area due to climate change (Dai, 2013). In this sense, water scarcity is becoming more frequent due to the overexploitation of aquifers as a result of an increasing demand (Dogan et al., 2016). This prevents the recovery of these sources of supply during recharge periods and it leads to the depletion of water resources. Agriculture is the largest user of water supplies, consuming over 70% of the abstracted freshwater globally (Singh, 2015). In recent years, wastewater reclamation, recycling and reuse has gained attention in many countries, to ensure water security and to develop effective strategies for sustainable utilization of water resources in agricultural and landscape irrigation (Mizyed, 2013; Pedrero et al., 2010; Bozdoğan, 2015; Saleem and Jabbar 2018). This kind of water is recovered from domestic, municipal, and industrial wastewater treatment plants and it may have specific treatments depending of its purpose (EPA: Guidelines for Water Reuse, 2012). Its use implies benefits of different nature, such as the pressure alleviation on other water resources (Eslamian et al., 2015) and the reduction of fertilizer cost due to its high nutrient content (Vivaldi et al., 2015), producing high-value crops and crop commodities (Silber et al., 2015). However, depending on its source and type of treatment, there is a wide range of chemical contaminants persisting in reclaimed wastewater, such as inorganic compounds, heavy metals, pathogens and many other complex compounds (Fatta-Kassinos et al., 2011), that may affect negatively crop yield or be unsafe to human consumption (Mara and Sleight, 2010; Intriago et al., 2018). In the case of plants with gardening and reforestation purposes, in addition to the problems related to the high salt concentrations and heavy metals which may affect growth, stabilization and quality of plants (Gómez-Bellot et al., 2015), the presence of persistent organic pollutants such as organochlorine pesticides can pose threats to ecosystems due to their biological accumulation through the trophic chain.

Some of these mentioned water treatments include membrane processes such as reverse osmosis (RO), whose main application is water desalination. Desalination technologies, particularly the reverse osmosis process, have been increasingly adopted to produce freshwater from alternative sources (Afrasiabi and Shahbazali, 2011). The water residue or RO brine resulting from this process is usually removed without using it, due to its high quantity of salts, causing environmental problems and high economic costs (Darre and Toor, 2018). This has led some authors to consider that the reuse of agricultural and industrial brines for crop production can be beneficial in preventing discharge of brines into natural environmental (Hamilton, 2004). Either way, there is a need to consider this water as a new non-potable water source (Nazer et al., 2018) and to improve brine management strategies (Jones et al., 2019). Numerous projects have been developed for the utilization of saline water on conventional crops and forages (Ayars and Soppe, 2004; Miyamoto, 2006; Romero-Trigueros et al., 2020). However, the low salinity tolerance of most crops limits the amount of saline water that can be applied for conventional crop production. In this sense, the selection and adoption of suitable plant species and genotypes are key factors to improve agricultural and green areas quality, as well as to decrease management costs. Halophytes plants are the native flora of saline soils, which survive completing their whole life cycle in such environments (Ksouri et al., 2019). Halophytes are not only used in landscaping or as ornamental plants, they are also used to treat saline effluence, or cultivated with industrial purposes. Last but not least, they are being used for forage/fodder, human food and even gourmet vegetables (Flowers and Colmer, 2015). The viability of plants in saline habitats depends on their ability to cope with several major constraints as (i) water deficit, (ii) restriction of CO_2 uptake, (iii) ion toxicity and (iv) nutrient imbalance (Riadh et al., 2010). Therefore, halophytic species may differ in their degree of salt tolerance (Flowers et al., 2015). To achieve this tolerance and be able to deal with salt stress, a wide range of morphological, physiological and biochemical mechanisms take place on the whole plant, at tissue and at cellular/molecular levels (Ashraf and Harris, 2004; Koyro et al., 2009).

The main salt tolerance target is keeping the ionic excess away from the metabolic active tissues to preserve leaf photosynthesis and meristematic activity (Moles et al., 2016). This aim is obtained generally thanks to processes such as accumulation of osmotic adjustment substances, ion-selective absorption and compartmentalization, morphological changes in root and leaf tissues, antioxidant and hormone regulation (Polic et al., 2009; Liang et al., 2018; Polash et al., 2019). Taking into account this last process, phytohormones emerge as cellular signal molecules with key functions in the regulation of plant responses to abiotic stresses. Recently, a considerable amount of evidence has shown that phytohormones are signals connecting root and shoot, triggering responses to external stress (Llanes et al., 2014). To avoid water losses due to evapotranspiration, plants regulate cell biophysics promoting cellular turgor decrease and leaf stomatal closure. Consequently, a reduction of stomatal conductance limits CO_2 uptake and photosynthesis ultimately (Chaves et al., 2009). The reduction in photosystem II efficiency by excess salinity is associated with decreasing total chlorophyll content. In salt tolerant plants, PSII photochemistry is reported to be more resilient to salt stress than CO_2 fixation processes, with a balance between the light-harvesting processes and effective energy dissipating mechanisms (Maricle et al., 2007; Moinuddin et al., 2017). Nevertheless, while halophytes are clearly capable of coping with excessive amounts of NaCl in their tissues (Flowers and Colmer, 2008; Shabala and Mackay, 2011), it is not clear whether they can tolerate other confounding factors and impurities associated with wastewater irrigation.

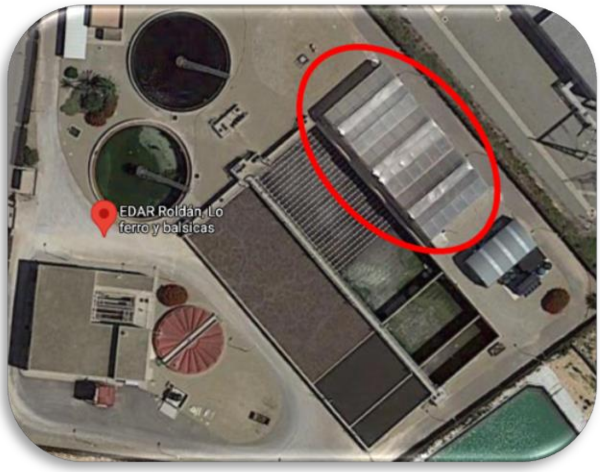
Crithmum maritimum (Apiaceae), or sea fennel, is a food halophyte found on rocky shores of Mediterranean Sea and Atlantic Ocean (Głoniak et al., 2006). Several uses of C. maritimum are known for culinary purposes and its leaves have been used for aromatic and medicinal purposes as a tonic and diuretic (Ruberto et al., 2000). C. maritimum is moderately tolerant to NaCl, known as a facultative halophyte, since it does not require salt for maximal growth. On the other hand, *Atriplex halimus*, or Mediterranean saltbush, is a xerohalophytic perennial shrub native to the Mediterranean. It is considered desirable due to its high fodder quality (Abbad et al., 2004) and due to its potential for use in ecological restoration programs (Soualem et al., 2018). A. halimus is well adapted to salinity by tolerating salts internally and/or by its excretition (Hassine et al., 2008) through its trichomes (Belkheiri and Mulas, 2013).

Based on the above considerations, in this study we evaluate if the irrigation with reclaimed wastewater and brine from a reverse-osmosis water treatment of two halophytes species (*Crithmum maritimum* and *Atriplex halimus*) growing on soil, is suitable for revegetation purposes. The objectives of the study were (i) to test the use of saline effluent such as brine from a reverse osmosis (RO) desalination treatment to irrigate *Crithmum maritimum* and *Atriplex halimus*, (ii) to study the growth of two halophyte forage species to factors associated with wastewater and (iii) to compare physiological traits, nutritional and hormonal status of two halophytes. Comparative data relating plant physiological and agronomic processes may prove beneficial information on the tolerance of plants to abiotic stresses.

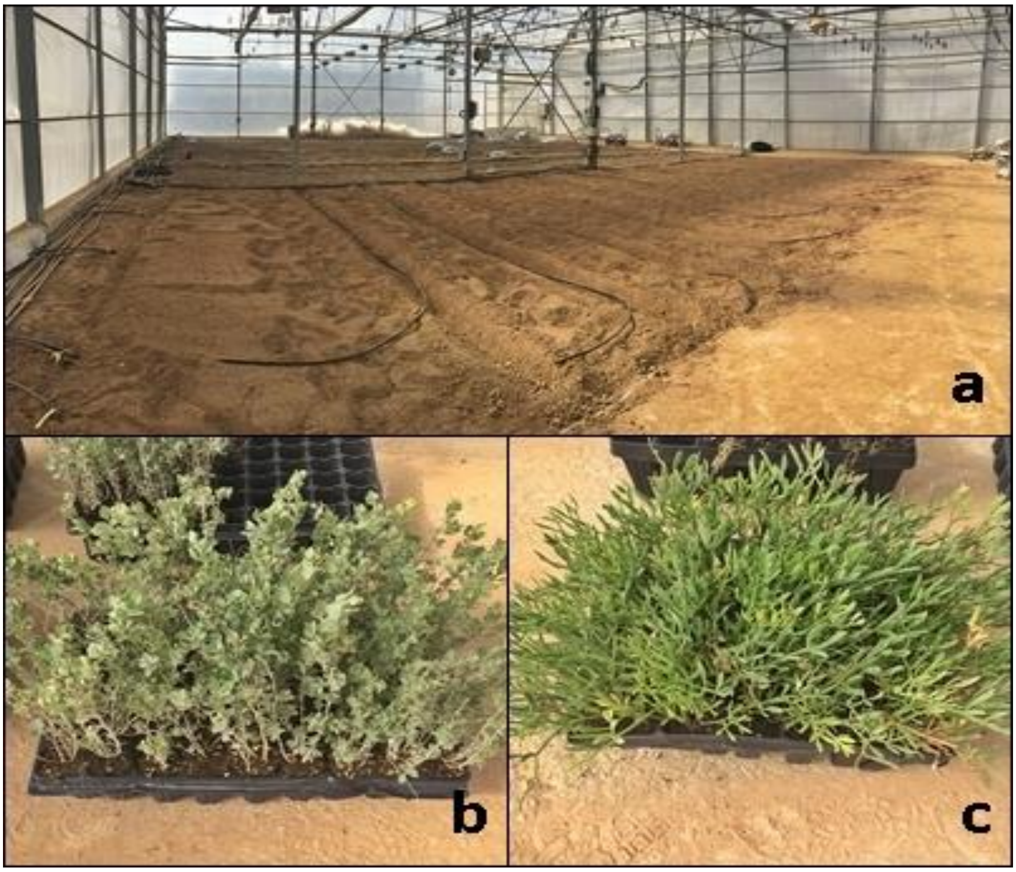
2. Material and Methods

2.1. Plant material and experiment conditions

The experiment was performed in a greenhouse located in the municipal wastewater treatment plant (WWTP) of Balsicas (Murcia, Spain) (latitude 37°47′48″ N, longitude 0°57′36″ W), from April 2018 to January 2019.



Picture 1. Greenhouse location.



Picture 2. Greenhouse interior (a), *Atriplex halimus* plants (b) and *Crithmum maritimum* plants (c).

Two halophyte species typical from Mediterranean silty clay loam soil. The average bulk density was 1.46 g cm^{-3} and the volumetric areas, *Crithmum maritimum* (CM) and *Atriplex halimus* (AH) were grown. Seedlings of both species (n = 72) were transplanted on 24 April 2018 into the greenhouse, which has a soil water content at field capacity and permanent wilting point were 36.3% and 19.8%, respectively. The experimental plot consisted of 6 rows, with a total length of approximately 6 meters and 12 plants per row, following a planting pattern of 0.5 × 1 m^2. The microclimatic conditions showed that during the experimental period, the average values of the air temperature, relative humidity and radiation were around 21 °C, 63% and 500 Wm^{-2}, respectively. Irrigation and agronomic management were established by the farmer. Plants were irrigated by a drip irrigation system with one lateral pipe per plant row and one emitter (3 L h^{-1}). The volume of water applied depended on the season, the climatic conditions and the plant development.

Irrigation was applied to bring soil moisture to the field capacity (up to a depth of 30 cm as the depth of root expansion). Soil moisture was measured using capacitive probes (ECHO-5, Decagon Devices Inc., Pullman, WA, USA) connected to a datalogger (CR1000, Campbell Scientific Inc., Logan, UT, USA). Irrigation was scheduled twice a week, activating it until soil moisture reached field capacity.

2.2. Irrigation water treatments and experimental design

Before starting with the different irrigation treatments, plants were irrigated with water from the Irrigation Community of Campo de Cartagena (< 0.9 dS m^{-1}). Irrigation treatments began on 23 May 2018, four weeks after transplanting. During the thirty-five following weeks, two irrigation treatments were applied at 100% field capacity: (1) Reclaimed wastewater (RWW) as control (EC: 0.8–1.2 dS m^{-1}), obtained by feeding wastewater to several tertiary treatments in the WWTP, such as ultrafiltration, granular activated carbon filter and four reverse osmosis membrane elements, and (2) reverse osmosis brine (ROB) (EC: 4.7–7.9 dS m^{-1}) which was a water residue result of the above mentioned process. The salinity level in the brine was medium, avoiding excessive salinization and soil degradation. Water quality of both treatments was similar during the experiment, it just varied in a narrow range, depending on the characteristics of the input wastewater treated at the WWTP (Table 1). In this sense, and in general terms, the concentration of the reverse osmosis brine components was much higher than those found in reclaimed wastewater. The concentration of Na, Cl and SO_4 was around six times higher in brine than in reclaimed wastewater, while the concentrations of anions such as F, NO_3 and PO_4 was around three times higher in brine than in reclaimed wastewater. Elements such as B, Ni, Cu and Zn showed a similar concentration in both waters (Table 1).

Table 1. Physicochemical analyses of the irrigation treatments. Data is presented as average values of the water samples collected during the experiment.

	RWW	**ROB**
EC (dS m^{-1})	0.994	5.403
pH	7.199	7.124
SS (mg L^{-1})	1.276	4.269
Turbidity (NTU)	0.570	0.686
E. coli (CFU 100 mL^{-1})	0.00	0.00
Fe (ppm)	0.04	0.07
K (ppm)	17.54	98.14
Mg (ppm)	8.45	56.12
Mn (ppm)	0.05	0.27
Na (ppm)	160.16	1003.29
Cl (ppm)	210.01	1208.92
P (ppm)	2.68	10.04
S (ppm)	1.88	9.11
B (ppm)	0.821	0.877
Ni (ppm)	0.008	0.008
Cu (ppm)	0.009	0.009
Zn (ppm)	0.054	0.044
F (ppm)	0.07	0.24
NO_2 (ppm)	0.10	0.10
NO_3 (ppm)	5.64	15.70
PO_4 (ppm)	8.21	30.72
SO_4 (ppm)	129.6	877.58

EC, electrical conductivity; SS, suspended solids; *E. coli*, *Escherichia coli* bacteria.

The treatments followed a randomized design, with three replications per treatment (6 plants per replication, 18 plants per treatment and species). Three rows were irrigated with RWW from the tertiary effluent and the other three with ROB in the plot.

2.3. Plant water relations

Leaf water relations were measured throughout the experiment in nine plants per treatment (three plants per replication). Leaf water potential (Ψ_{leaf}) was measured at midday, collecting a mature leaf according to Scholander et al. (1965) using a pressure chamber (Model 3000; Soil Moisture Equipment Co., Santa Barbara, CA, USA). Leaves were placed in the chamber within 20 s of collection and pressurized at a rate of 0.02 MPa s^{-1} (Turner, 1988). Adjacent leaves were also collected, frozen immediately in liquid nitrogen (−196 °C) and subsequently stored at −30 °C. After thawing, the leaf osmotic potential (Ψ_{os}) was measured in the extracted sap using a WESCOR 5520 vapor pressure osmometer (Wescor Inc., Logan, UT, USA), according to Gucci et al. (1991) The leaf osmotic potential at full turgor (Ψ_{100s}) was estimated as indicated above for Ψ_{os}, after placed in distilled water overnight to reach full saturation. The leaf turgor potential (Ψ_t) was estimated as the difference between leaf water potential (Ψ_{leaf}) and leaf osmotic potential (Ψ_{os}).

Picture 3. WESCOR 5520 vapor pressure osmometer (Wescor Inc., Logan, UT, USA).

2.4. Gas exchange and chlorophyll fluorescence parameters

Leaf gas exchange and chlorophyll fluorescence were measured simultaneously at midday throughout the experiment using a gas exchange system (LI-6400; LI-COR Inc., Lincoln, NE, USA), fitted with an infrared gas analyzer attached to a leaf chamber fluorometer (LCF) (6400-40B, 2 cm^2 leaf area, Licor Bioscience, Inc., Lincoln, NE, USA). The reference CO_2, photosynthetically active radiation (PAR) and speed of the circulating air flow inside the system were set at 400 ppm, at 2000 $\mu mol\ m^{-2}\ s^{-1}$ and at 500 $\mu mol\ s^{-1}$, respectively. The leaf photosynthetic rate (P_n), stomatal conductance (g_s), internal CO_2 concentration (C_i), the excitation capture efficiency of open centres (F_v'/F_m'), the effective quantum efficiency of photosystem II (Phi PSII), photochemical quenching coefficient (qP) and the electron transport rate (ETR) were measured (Mashilo et al., 2018). The intrinsic water use efficiency (WUE_i) was determined as the P_n/g_s ratio, and the photosynthetic efficiency was expressed as the relationship between the degree of stomatal opening necessary to reach a certain level of photosynthesis.

2.5. Qualitative and quantitative analysis of phytohormones and chlorophyll content in leaves

Analytical standards of the phytohormones 1-aminocyclopropane-1-carboxylic acid, giberellic-5 acid, trans-Zeatin glucoside, abscisic acid, salicylic acid and scopoletin were purchased from Santa Cruz Biotechnologies (Dallas, TX, USA). trans-Zeatin, trans-Zeatin riboside and [2H5]-trans-Zeatin were obtained from OlchemIm (Olomouc, Czech Republic). Ethanol, Water LC-MS quality, dimethyl sulfoxide, formic acid and methanol were bought from Panreac (Barcelona, Spain). Acetonitrile was from J.T. Baker (Thermo Fisher Scientific Inc., Waltham, MA, USA).

For the identification and quantification of hormones, 0.1 g of fresh leaves from 6 samples per treatment were crushed in a mortar with liquid nitrogen and stored at −80 °C. Then, they were vortexed with 0.5 mL 80% methanol/water (v/v) and incubated at 4 °C during 30 min and finally centrifuged at 15,000 rpm (20,627× *g*), at 4 °C for 15 min. The supernatant was kept in ice and then it was further extracted with 0.5 mL 80% methanol/water (v/v) after being incubated and centrifuged under the same conditions described above. Finally, both supernatants from the two previous extractions were passed through Chromafix C_{18} solid phase extraction cartridge (Macherey Nagel, Düren, Germany) (previously activated with 3 mL 80% methanol/water (v/v). The eluted sample was concentrated to dryness by the use of a rotary vacuum evaporator during approximately 3 h (Speedvac, Thermo, Waltham, MA, USA). Then, the dry residue was resuspended with 200 µL de 20% metanol/water (v/v), sonicated for 8 min and filtrated through 0.45 µm polyethersulfone filter (Millipore) and finally injected in a ultra-high-performance liquid chromatography (UHPLC) coupled triple quadrupole mass spectrometry (UHPLC-ESI-QqQ-MS/MS) for qualitative and quantitative analysis (Rivier and Crozier, 1987).

Chromatographic separation of phytohormones and the phytoalexin scopoletine was performed by a method previously described by Albacete et al., (2008) with slight modifications. Briefly, we used a UHPLC coupled to a 6460 UHPLC-ESI-QqQ-MS/MS (Agilent Technologies, Waldbronn, Germany), using a BEH C_{18} analytical column (2.1 × 100 mm, 1.7 µm) (Waters, Milford, MA, USA). Mobile phases A (H_2O) contained 0.01% formic acid (v/v) and B acetonitrile. The flow rate was 0.2 mL/min using a linear gradient scheme: (t; %B): (0.0; 19.00), (2.5; 90.00), (4.5; 90.00), (6.00; 19.00), (8.00; 19.00). The injection volume was 10 µL. The column temperatures were 40 °C. The operating conditions for the ionization source were as follows: Gas flow: 8 L/min, Nebulizer: 45 psi, Capillary Voltage: 4000 V (positive mode) and 2750 V (negative mode), Nozzle Voltage: 1000 V (positive mode) and 1500 V (negative mode), Gas Temperature: 300 °C, Sheath Gas Temperature: 375 °C and Jetstream Gas Flow: 11 L/min. The ion optics and fragmentation conditions are detailed in Table 2. Data acquisition and processing were performed using Mass Hunter software version B.08.00 (Agilent Technologies). The quantification of the phytohormones and scopoletin detected in the samples was performed according to standard curves freshly prepared each day of analysis.

Table 2. Ultra-high-performance liquid chromatography (UHPLC) coupled triple quadrupole mass spectrometry (UHPLC-QqQ-MS/MS) parameters for the identification, identity confirmation (second MRM transition) and quantification of the phytohormones.

Hormone [Z]	Retention Time (min)	Ionization Mode	Parent Ion (m/z)	Ion Fragments (m/z)	Fragmentor (V)	Collision Energy (V)
ACC	1.312	Positive	102.1	56.0 [Y]	80	15
				28.0 [X]	80	15
tZ	1.724	Positive	220.2	202.0	80	15
				136.0	80	15
tZdeuter	1.744	Positive	225.2	136.3	80	15
tZ-GLC	1.742	Positive	382.4	220.0	80	15
				202.0	80	15
tZ-Rib	1.743	Positive	352.4	219.7	80	15
				136.0	80	15
SC	2.802	Positive	193.2	132.5	80	20
				149.1	80	20
GA5	3.095	Negative	329.4	145.0	80	39
				285.0	80	18
ABA	3.130	Negative	263.3	152.9	80	14
				204.1	80	18
SA	3.219	Negative	137.1	93.2	80	15
				65.4	80	15

[Z] ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; GA5, giberellic-5 acid; SA, salicylic acid; SC, scopoletin; tZ, trans-zeatin; Tz-Glc, trans-zeatin glucoside; Tz-Rib, trans-zeatin riboside. [Y] MRM transition for quantification. [X] MRM transition for confirmation.

At the end of the experiment, the chlorophyll content was assayed according to Inskeep and Bloom (1985) in the leaves of four plants per treatment. The extraction was made from 50 mg of fresh material in 5 mL of 80% acetone in the dark at 4 °C. The extract was read at 647 nm for chlorophyll-a, and 664 nm for chlorophyll-b in an Uvikon 940 spectrophotometer (Kontron Instruments AG, Zürich, Switzerland).

2.6. Determination of mineral content in leaves and plant canopy

The inorganic mineral content of dry leaves was determined at the end of the experiment in three plants per treatment (one sample per replication) by means of emission spectrophotometry. The leaves were oven dried at 80 °C, ground, and sieved through a 2-mm nylon mesh before analysis. A chemical analysis of water irrigation treatments was performed. The nutrient concentrations were determined in an extract digested with HNO_3:$HClO_4$ (2:1, v/v) using an inductively coupled plasma optical emission spectrometer (ICP-OES IRIS INTREPID II XDL). At the end of the experiment, the plant canopy was determined in both species by measuring height and width from the top, selecting nine representative plants per treatment (three plants per replication).

2.7. Statistics

In the experiment, all plants (n = 72) were randomly assigned to each treatment, with three replications for each treatment. The data were analyzed by one-way ANOVA and two-way ANOVA using IBM SPSS Statistics 25. The independent variables were irrigation water and species. Treatment means were separated with Duncan's multiple range test ($P \leq 0.05$).

3. Results

3.1. Plant water relations

Throughout the experiment, the highest values of leaf water potential and osmotic water potential were found in *Crithmum* plants, with no differences in the osmotic water potential by the type of water. The lowest values of these parameters were found in *Atriplex* plants, especially in those irrigated with ROB (Figure 1A,B). *Atriplex* plants showed also the lowest values of osmotic potential at full turgor during the experiment (Figure 1C). In general, leaf turgor potential was higher in *Atriplex* plants than in *Crithmum*, while there were differences only by the irrigation type at the beginning of the experiment, ROB treatment showing higher values than RWW treatment (Figure 1D).

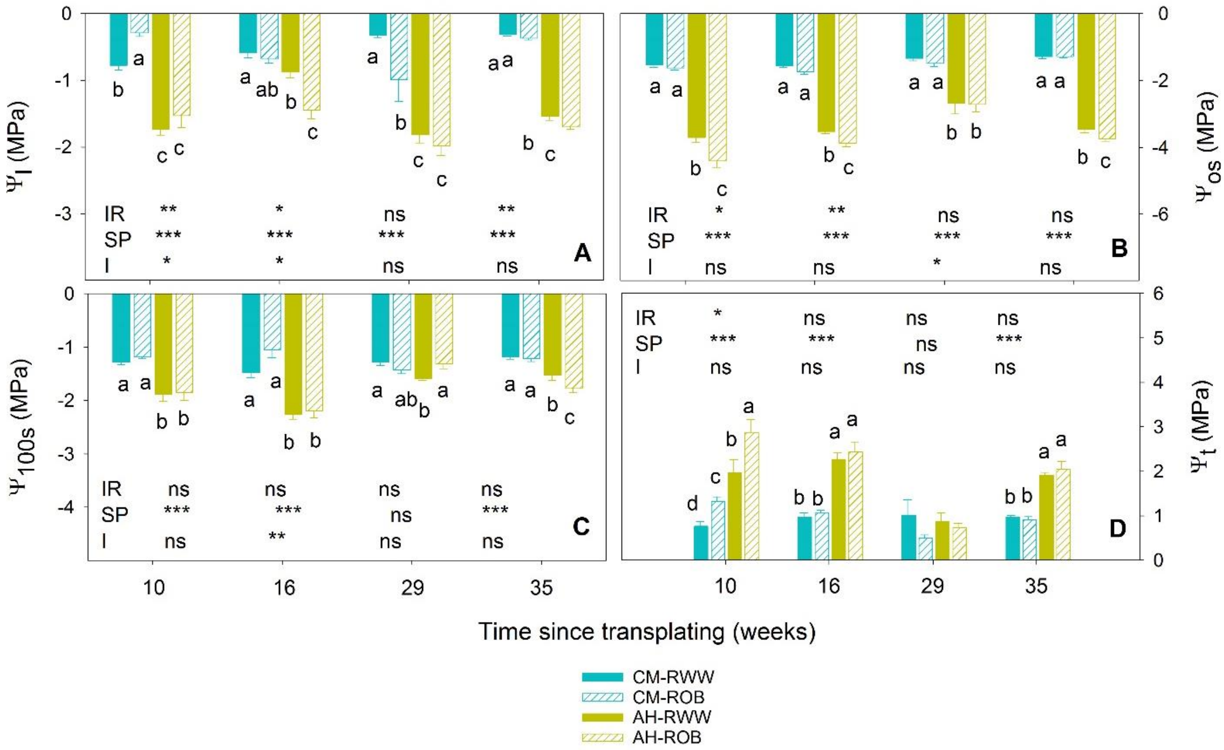


Figure 1. Leaf water potential (Ψ_{leaf}) (**A**), osmotic water potential (Y_{os}) (**B**), osmotic water potential at full turgor (Ψ_{100s}) (**C**) and leaf turgor potential (Ψ_t) (**D**) in *Crithmum maritimum* (CM) and *Atriplex halimus* (AH), irrigated with reclaimed wastewater (RWW) and reverse osmosis brine (ROB). Results were from one way and two-way ANOVA tests in these parameters, for independent variables (irrigation, IR, and species, SP) and their interaction (I). Different lowercase letters indicate significant differences between treatments according to Duncan's test at $p \leq 0.05$. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. $p > 0.05$ non-significant differences are indicated by "ns".

3.2. Gas echange and chlorophyll fluorescence parameters

In both species, the irrigation with ROB induced a decrease in g_s compared with the RWW treatment during the first half of the experiment, showing *Atriplex* irrigated with ROB the lowest values (Table 3). In fact, regardless the type of irrigation, the lowest g_s values were found in *Atriplex*. Nevertheless, there were no differences in P_n by irrigation type, except at the beginning of the experiment (week 10), when irrigation with ROB decreased P_n in both species, being this decrease only significant for *Atriplex* plants (Table 3). Regardless the type of water used, *Atriplex* showed the highest values of P_n at week 12 and 16, while *Crithmum* showed the highest ones at the end of the experiment. The intrinsic water use efficiency (WUEi) barely experienced variations resulting from the type of irrigation water. Despite these unclear variations, in general terms, *Atriplex* plants showed a higher WUEi than *Crithmum* (Table 3).

Table 3. Stomatal conductance (g_s), net photosynthetic rate (P_n), and intrinsic water use efficiency (WUE_i) in *Crithmum maritimum* (CM) and *Atriplex halimus* (AH), irrigated with reclaimed wastewater (RWW) and reverse osmosis brine (ROB). Results were from one-way and two-way ANOVA tests on these parameters for independent variables (Irrigation, IR, and species, SP) and their interaction (I). Values are means of nine samples.

g_s (mmol m^{-2} s^{-1})					
Week	**10**	**12**	**14**	**16**	**19**
CM-RWW	268.4 a	210.9 a	199.2 b	110.5	333.3 a
CM-ROB	211.9 ab	177.3 ab	299.1 a	122.8	285.4 ab
AH-RWW	152.8 bc	123.6 bc	120.4 bc	94.1	204.4 b
AH-ROB	105.0 c	58.4 c	95.5 c	92.4	196.7 b
Sig.	**	**	***	ns	*
IR	*	*	ns	ns	ns
SP	**	**	***	ns	**
I	ns	ns	*	ns	ns
P_n (µmol m^{-2} s^{-1})					
Week	**10**	**12**	**14**	**16**	**19**
CM-RWW	16.93 a	10.47 b	10.76	6.587 b	26.69 a
CM-ROB	12.90 ab	9.56 b	14.10	7.104 b	24.18 ab
AH-RWW	18.82 a	16.89 a	14.52	14.68 a	18.21 b
AH-ROB	10.17 b	15.60 a	11.64	14.42 a	17.57 b
Sig.	*	**	ns	***	*
IR	***	ns	ns	ns	ns
SP	ns	***	ns	***	**
I	*	ns	ns	ns	ns
WUEi (P_n/g_s)					
Week	**10**	**12**	**14**	**16**	**19**
CM-RWW	69.0 b	51.4 b	54.8 b	62.2 b	80.9
CM-ROB	65.3 b	105.8 b	50.2 b	60.2 b	87.6
AH-RWW	119.1 a	157.1 b	113.6 a	157.6 a	92.2
AH-ROB	91.0 b	369.6 a	120.0 a	155.9 a	93.5
Sig.	***	**	***	***	ns
IR	*	*	ns	ns	ns
SP	***	**	***	***	ns
I	*	ns	ns	ns	ns

Different lowercase letters indicate significant differences between treatments according to Duncan's test at $p \leq 0.05$. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. $p > 0.05$ non-significant differences are indicated by "ns".

The intercellular CO_2 (C_i) in both species was hardly affected by the type of water used during the experiment (Figure 2A).

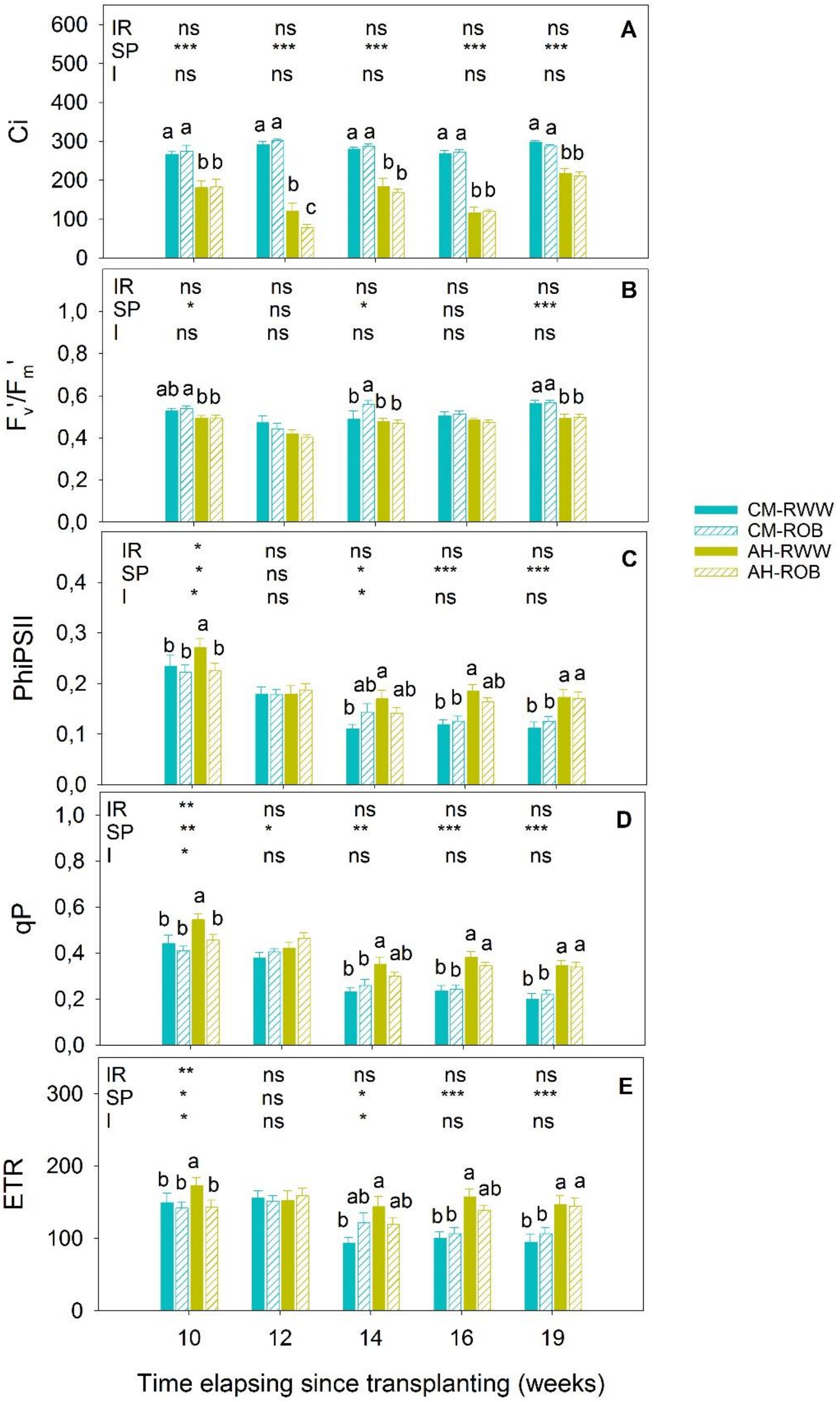


Figure 2. Internal CO_2 concentration (C_i) (**A**); The excitation capture efficiency of open centers (F_v'/F_m') (**B**); PSII effective quantum yield (PhiPSII) (**C**); Photochemical quenching coefficient (qP) (**D**); and the apparent electron transport rate (ETR) (**E**) in *Crithmum maritimum* (CM) and *Atriplex halimus* (AH) irrigated with reclaimed wastewater (RWW) and reverse osmosis brine (ROB).

Results were from one way and two-way ANOVA tests in these parameters, for independent variables (irrigation, IR, and species, SP) and their interaction (I). Different lowercase letters indicate significant differences between treatments according to Duncan's test at $p \leq 0.05$. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. $p > 0.05$ non-significant differences are indicated by "ns".

Regardless the irrigation type, *Crithmum* showed higher C_i values than *Atriplex*. No statistical differences in $F_{v'}/F_{m'}$ were observed by the type of water used during the experiment, while irrespective of the irrigation type, *Crithmum* showed the highest values throughout almost the whole experiment (Figure 2B). Regardless the type of water used, *Atriplex* showed increased PhiPSII, qP and ETR values most of the weeks (Figure 2C–E). The photosynthetic efficiency showed a similar trend line for both irrigation treatments in *Crithmum*, since for the same photosynthesis value, the stomatal conductance was similar, although from 12 μmol m^{-2} s^{-1} of P_n approximately, plants irrigated with ROB showed a g_s slightly higher (Figure 3A). In *Atriplex*, plants irrigated with ROB had a lower photosynthetic efficiency than those irrigated with the RWW, since from 13 μmol m^{-2} s^{-1} of P_n approximately, for the same P_n value, the stomatal conductance of plants irrigated with ROB was higher (Figure 3B).

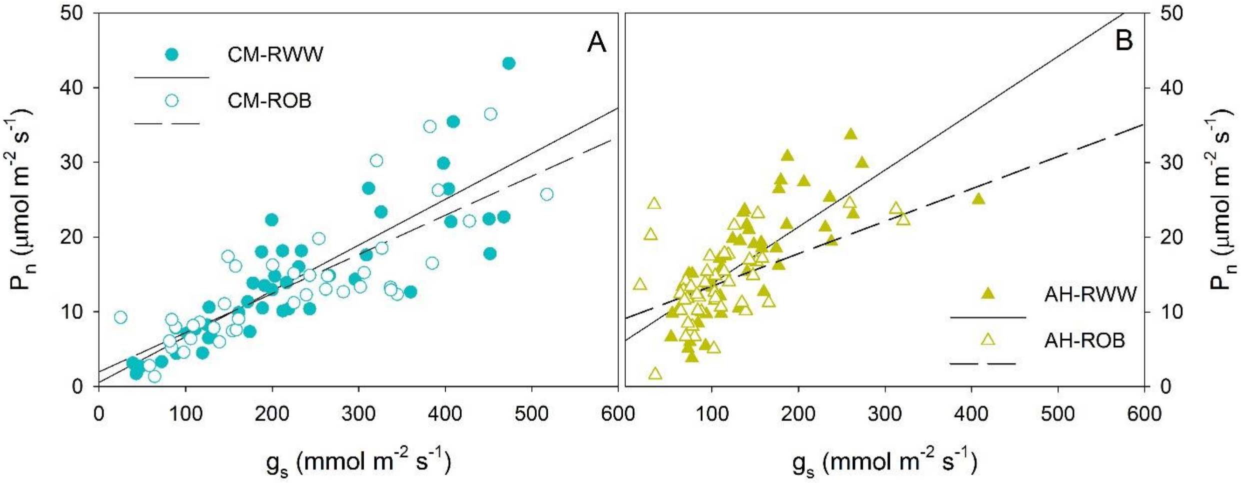


Figure 3. Photosynthetic efficiency as the relationship between photosynthesis and stomatal conductance in *Crithmum maritimum* (CM) (A) and *Atriplex halimus* (AH) (B) irrigated with reclaimed wastewater (RWW) and reverse osmosis brine (ROB).

3.3. Phytohormones and chlorophyll content in leaves

The leaf chlorophyll content was not affected by the type of water although the highest values were found in *Atriplex* (Table 4).

Table 4. Leaf chlorophyll content (Chl A, Chl B and Chl T) in *Crithmum maritimum* (CM) and *Atriplex halimus* (AH), irrigated with reclaimed wastewater (RWW) and reverse osmosis brine (ROB). Results were from one-way and two-way ANOVA tests on these parameters for independent variables (Irrigation, IR, and species, SP) and their interaction (I). Values are means of four samples.

	Leaf Clophyll Content (mg m^{-3})		
	Chl A	**Chl B**	**Chl T**
CM-RWW	0.539 b	0.171 b	0.710 b
CM-ROB	0.719 b	0.242 b	0.961 b
AH-RWW	2.048 a	0.549 a	2.597 a
AH-ROB	2.107 a	0.556 a	2.663 a
Sig.	***	**	***
IR	ns	ns	ns
SP	**	*	*
I	ns	ns	ns

Different lowercase letters indicate significant differences between treatments according to Duncan's test at $p \leq 0.05$. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. $p > 0.05$ non-significant differences are indicated by "ns".

Several phytohormones were identified in leaves in both species: the precursor of Ethylene, 1-Aminocyclopropane-1-carboxylic acid (ACC), cytokinins (TZ, TZ-rib and TZ-glc), gibberellins (GA5), abscisic acid (ABA), salicylic acid (SA) and the phytoalexin scopoletin (SC).

Some of them (ACC, TZ-rib and TZ-glc) were modified by the type of irrigation water. However, they were affected differently depending on the species. A higher ACC and TZ-glc concentration and lower ABA concentration were observed in *Crithmum* plants irrigated with ROB compared with those irrigated with water from RWW (Figure 4). *Atriplex* plants irrigated with ROB showed lower concentration of TZ-rib and SC compared with those irrigated with water from RWW (Figure 4). Regarding only species, higher values of ACC, TZ-glc and SA were found in *Crithmum* than in *Atriplex* plants, while higher values of TZ, GA5, ABA and the phytoalexin SC were found in *Atriplex* than in *Crithmum* (Figure 4).

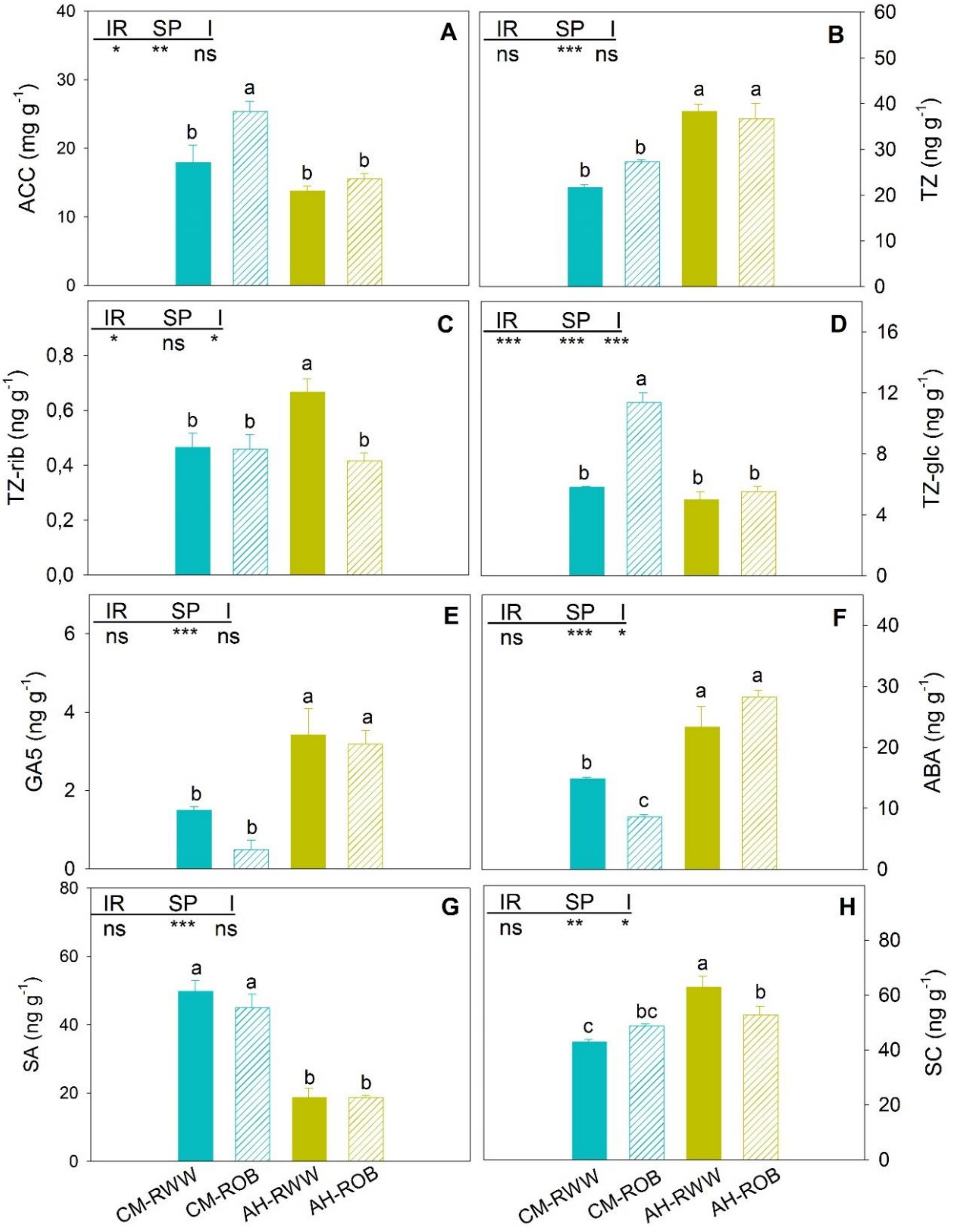


Figure 4. 1-Aminocyclopropane-1-carboxylic acid (ACC) (**A**), cytokinins (TZ) (**B**), (TZ-rib) (**C**), (TZ-glc) (**D**), gibberellins (GA5) (**E**), abscisic acid (ABA) (**F**), salicylic acid (SA) (**G**) and scopoletin (SC) (**H**), in *Crithmum maritimum* (CM) and *Atriplex halimus* (AH) irrigated with reclaimed wastewater (RWW) and reverse osmosis brine (ROB). Results were from one way and two-way ANOVA tests in these parameters, for independent variables (irrigation, IR, and species, SP) and their interaction (**I**). Different lowercase letters indicate significant differences between treatments according to Duncan's test at $p \leq 0.05$. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. $p > 0.05$ non-significant differences are indicated by "ns".

3.4. Leaf mineral concentration and plant canopy development

Regardless the species, the concentrations of B, Ca, Fe, K, Mg, Na, P and Zn ions in leaves did not show any significant changes by the type of water used (Table 5). Only B concentration in *Crithmum* leaves was lower after irrigating with ROB. Regardless the type of water, a higher amount of B and Ca ions accumulated in *Crithmum* leaves than in *Atriplex*, and a higher amount of K, Mg, Na and Zn accumulated in *Atriplex* leaves than in *Crithmum* (Table 5).

Table 5. Leaf mineral concentration in *Crithmum maritimum* (CM) and *Atriplex halimus* (AH), irrigated with reclaimed wastewater (RWW) and reverse osmosis brine (ROB). Results were from one-way and two-way ANOVA tests on these parameters for independent variables (irrigation, IR, and species, SP) and their interaction (I). Values are means of three samples.

ppm	**B**	**Ca**	**Fe**	**K**	**Mg**	**Na**	**P**	**Zn**
CM-RWW	329.0 a	45143.3 a	126.5	34673.3 b	3497.0 b	24931.7 b	3313.2	31.1 b
CM-ROB	263.5 b	44260.0 a	112.9	19582.8 b	3343.0 b	17809.2 b	3324.8	40.4 ab
AH-RWW	248.9 b	11013.3 b	92.6	76266.7 a	9628.3 a	56661.7 a	3218.5	52.2 a
AH-ROB	248.0 b	9726.7 b	94.5	65616.7 a	9315.0 a	71816.7 a	2879.2	50.1 a
Sig.	*	*	ns	**	***	**	ns	ns
IR	ns	ns	ns	ns	ns	ns	ns	ns
SP	*	**	ns	***	***	***	ns	*
I	ns	ns	ns	ns	ns	ns	ns	ns

Different lowercase letters indicate significant differences between treatments according to Duncan's test at $p \leq 0.05$. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. $p > 0.05$ non-significant differences are indicated by "ns".

At the end of the experiment, the structure of the plant or its canopy was not statistically affected by the type of water used (Table 6). *Atriplex* showed a slight reduction of the canopy caused by the irrigation with brine but with no significant differences, while *Crithmum* reached numerically greater canopy by the irrigation with ROB, but not statistically. Regardless the type of water used, *Atriplex* plants developed a greater canopy than *Crithmum* (Table 6).

Table 6. Plant canopy determined by measuring height and width of plants in *Crithmum maritimum* (CM) and *Atriplex halimus* (AH), irrigated with reclaimed wastewater (RWW) and reverse osmosis brine (ROB). Results were from one-way and two-way ANOVA tests on these parameters for independent variables (irrigation, IR, and species, SP) and their interaction (I). Values are means of three samples.

	CANOPY (cm)		
	Height (H)	**Width (W)**	**H × W**
CM-RWW	24.44 b	28.94 b	770.50 c
CM-ROB	28.72 b	33.94 b	1008.17 c
AH-RWW	65.94 a	104.56 a	6880.00 a
AH-ROB	57.72 a	97.72 a	5723.67 ab
Sig.	***	***	***
IR	ns	ns	ns
SP	***	***	***
I	ns	ns	ns

Different lowercase letters indicate significant differences between treatments according to Duncan's test at $p \leq 0.05$. *** $p < 0.001$. $p > 0.05$ non-significant differences are indicated by "ns".

4. Discussion

Crithmum maritimum and *Atriplex halimus* are naturally salt-tolerant plants, being great candidates to replace conventional edible sensitive plants in marginal and degraded lands (Jalali et al., 2012). *Atriplex* may even be useful for phytoremediation of former mining areas (Lefèvre et al., 2009). Although the salinity range in our experiment was moderately low, the use of reclaimed wastewater and brine has scarcely been proved in these plants. It is not clear whether they can tolerate other confounding factors and impurities associated with wastewater (Xu and Drewes, 2006). Regarding water relations, both species performed differently to the use of saline effluent from reverse osmosis (RO) brine. The reduction of leaf water potential by the irrigation of ROB was more evident in *Atriplex* plants, which reduced the osmotic leaf potential to maintain leaf turgor values similar to those plants irrigated with water from RWW. When water potential is suddenly reduced, osmotic adjustment occurs rapidly to allow partial turgor recovery and re-establishment of water potential gradient for water uptake, and the loosening ability of the cell wall increases (Sucre and Suárez, 2011). Many plants accumulate organic osmolytes in their cytoplasm (Negrão et al., 2017) to increase cellular water retention without affecting normal metabolic processes. However, *Crithmum* plants did not need to reduce the osmotic potential. ROB water seemed to decrease gas exchange, especially g_s, in both plant species respect to the water from RWW during the first half of experiment, *Atriplex* in a greater extent. However, leaf chlorophyll content was not affected by the type of irrigation water, most halophyte plants are able to stabilize chloroplasts and thylakoids to protect photosynthesis mechanisms (Slama et al., 2015). Little literature has been found about the role of the photosynthetic activity in these species (Labidi et al., 2011; Benzarti et al., 2012). Benzarti et al. (2012) observed that g_s in *Atriplex portulacoides* was only reduced above 200 Mm (≈18 dS m^{-1}) NaCl in the water. Although there were hardly any changes in the fluorescence parameters and WUEi by the type of water in these plants, the photosynthetic efficiency results indicated that *Atriplex* plants irrigated with RO brine needed to open more their stomas than those irrigated with RWW, to reach the same level of P_n. As a consequence, *Atriplex* plants had a greater loss of water and higher energy costs than *Crithmum* (Sikder et al., 2015). The greater concentration of mineral ions in brine water than in water from RWW, such as K, Mg, Na, S and P, did not lead to a higher leaf ion accumulation in both species. Nevertheless, each plant species had a different preference to accumulate ions in their leaves. *Crithmum* accumulated higher B and Ca content, the latter transported to the leaves by the transpiration process (Gilliham et al., 2011), suggesting a better flow of water to leaves, while *Atriplex* accumulated higher K, Mg, Na and Zn content. This indicated that each plant species had different mechanisms of ion uptake. In addition, both irrigation waters presented levels of heavy metals within the recommended concentration limits, although levels of salts such as Na, Cl and SO_4 were considered toxic for most crops (Fipps, 2003; Bauder et al., 2011; Malakar et al., 2019).

Phytohormones have also important roles in salt stress tolerance (Tuteja, 2007; Cao et al., 2007). The biosynthesis of ethylene is induced by many stresses, however, its role is controversial regarding salt stress (Wang et al., 2011). Khan et al. (2012) reported that increased ethylene biosynthesis in wheat was related to salt tolerance, while other authors claimed that its production might play a negative role in tomato growth, coinciding with an oxidative stress and leaf senescence (Albacete et al., 2009; Ghanem et al., 2008). In our experiment, the increase of ACC in *Crithmum* plants irrigated with ROB was not related to a negative response of plant physiology, since there was no evidence of oxidative stress or reduction of plant growth. A significant accumulation of ABA is essential to active plant protective mechanisms (Shakirova et al., 2010), which regulate leaf water potential and stomatal closing to avoid water losses (Tombesi et al., 2015). It is well known that the increase of ABA synthesis and the decrease of TZ production is an effective defense mechanism of plants in response to salt stress (Yu et al., 2020). However, in our experiment this behavior was not so clear in *Crithmum* plants, since RO brine did not induce the accumulation of ABA. *Atriplex* performed differently to *Crithmum*, since a lower leaf accumulation of TZ-rib and SC was observed in plants irrigated with brine compared to those irrigated with water from RWW. Scopoletin (phytoalexin) is a coumarin compound with antifungal properties and inhibitory effects on abiotic stresses (Gnonlonfin et al., 2012). Its accumulation has been correlated with resistance to stresses, such as dehydration and salt toxicity (Gnonlonfin et al., 2012; Tanaka et al., 1983). This fact might explain that the plants did not suffer salt stress or that salinity was not high enough to cause the accumulation of SC. Some studies showed that *Crithmum maritimum* is able to maintain growth at high salinity levels, even up to 340 mM NaCl (Hamdani et al., 2017; Jiménez-Becker et al., 2019). However, Ben Hamed et al. (2004) found that DW biomass of *Crithmum* begun to reduce even at 50 (4.5 dS m^{-1}) mM NaCl. In our case, the growth of both species was not affected by the different irrigation treatments, despite that the brine was 5.4 dS m^{-1} on average. In the *Atriplex* plants, irrigation with brine slightly reduced the canopy, surely due to lower photosynthetic efficiency showed in these plants. The physiological adaptations to salinity were more evident in *Atriplex* than in *Crithmum* plants to maintain growth, when they were irrigated with RO brine. Nedjimi and Daoud (2006) and Boughalleb and Denden (2011) showed an optimal plant growth in *Atriplex* at 50 mM Na_2SO_4, and at 100 mM NaCl, respectively, declining with a further increase in salinity.

5. Conclusions

In general, both species had a different physiological and nutritional response when they were irrigating with ROB. In the case of *Atriplex*, plants performed adaptations such as osmotic adjustment and stomatal regulation to maintain growth in comparison with *Crithmum*. In *Crithmum* plants irrigated with ROB, water status and photosynthetic activity performance, including photosynthetic efficiency, were similar to those irrigated with RWW. Nevertheless, the particular characteristics associated to this kind of waters did not seem to influence negatively the development of both species during the stabilization period. Therefore, reclaimed wastewater from tertiary effluence and reverse osmosis brine, such as the wastewater used in this experiment, could be an alternative to irrigation with conventional waters in both species for revegetation or soil preservation purposes.

GENERAL CONCLUSIONS

Chapter 1. The contribution of mycorrhizae in Cistus albidus plant growing with different types of substrate and submitted to water stress.

- Cistus plants growing in compost substrate have a good nutritional balance, although, when exposed to water stress, they suffer more than plants grown in commercial substrate due to the presence of heavy elements, which negatively affect growth.
- Leaf anatomy of cistus plant changed according to the substrate and the imposed water stress level. In the commercial substrate, the thickness of the leaf tissue decreased, while in the compost substrate, the thickness of the adaxial epidermis and parenchymal tissues increased.
- The development of water stress was slower in cistus plants growing in compost substrate due to limitations in their transpiration process. The restoration of irrigation represented a new stress for the plants, especially those growing in commercial substrate.
- Cistus plants adapt to water stress by decreasing aerial growth and increasing water use efficiency. Water stress positively stimulated the levels ABA and SA, strongly enhancing drought tolerance.
- *Cistus albidus* can form both ectomycorrhizae (ECM) and arbuscular mycorrhizal fungus (AMF) under controlled growth chamber conditions and in pots with different substrates.
- *Glomus iranicum* var. *tenuihypharum* is compatible with *Cistus albidus* in compost and commercial substrates, minimizing Na ion accumulation and increasing K and P levels in leaves.
- *Pisolithus tinctorius* inoculation in *Cistus albidus* improves plant growth and alleviates the harmful effects of drought, since leaf scopoletin levels are reduced.

Chapter 2. Suitability strategies for the use of saline water for irrigation in different species.

- *Asteriscus maritimus* and *Salvia officinalis* are susceptible to mycorrhization with *Glomus iranicum* var. *tenuihypharum*, which improves the photochemical parameters in both species when they are irrigated with saline reclaimed water.
- The higher salinity tolerance of *Asteriscus maritimus* to irrigation with reclaimed wastewater is due to it retaining toxic ions in roots, hindering their absorption and limiting their accumulation in the aerial part.
- In *Asteriscus maritimus* and *Salvia officinalis*, the return to fresh water irrigation induces the recovery of gas exchange, water status and the ability of plants to repair membrane damage.
- *Atriplex halimus* plants irrigated with reverse osmosis brine (ROB) maintained growth through osmotic adjustment and the regulation of stomatal activity. In *Crithmum maritimum*, ROB irrigation did not influence the water relations or the photosynthetic activity.
- Irrigation with reclaimed wastewater and reverse osmosis brine can be regarded as a good alternative to using conventional irrigation water for revegetation purposes. The effects of salinity did not visibly influence the development of *Atriplex halimus* or *Crithmum maritimum.*

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ABBREVIATIONS AND SYMBOLS

Abbreviations

ABA	Abscisic acid
AbE	Abaxial epidermis
$Abs_{664.5}$	Absorbance at 664.5 nm
Abs_{647}	Absorbance at 647 nm
ACC	1-aminocyclopropane1-carboxylic acid
AdE	Adaxial epidermis
AH	*Atriplex halimus*
AMF	Arbuscular mycorrhizal fungi
ANOVA	Analysis of variance

C	Commercial substrate
C	Control
C-	Control plants
C+	Inoculated Control plants
CEBAS	Centro de Edafología y Biología Aplicada del Segura
CFU	Colony-forming unit
Chl A	Chlorophyll a
Chl B	Chlorophyll b
Chl_T/Chl T	Chlorophyll total
C_i	Internal CO_2 concentration
CK	Citokinin
Cm	Centimetre
CM	*Crithmum maritimum*
CM	Inoculated plants in commercial substrate
CM	Inoculated well-watered control plants
CMD	Water-stressed inoculated plants in commercial substrate
CO_2	Carbon dioxide
Cp	Compost substrate
CpM	Inoculated compost substrate
CpMD	Water-stressed inoculated plants in compost substrate
CpWS	Water-stressed plants in compost substrate
CpWW	Well-watered plants in compost substrate

CSIC	Consejo Superior de Investigaciones Científicas
CWS	Water-stressed plants in commercial substrate
CWW	Well-watered plants in commercial substrate
DMF	Dimethylformamide
dS	Decisiemen
DW	Dry weight
EC	Electrical conductivity
ECM	Ectomycorrhizae fungus
ESI	Electrospray interface
ET	Ethylene
ETR	Electron transport rate
F_v/F_m	Maximal photochemical efficiency of photosystem II
F_v'/F_m'	Excitation capture efficiency of open centres
FW	Fresh weight
g	Gram
g	Relative centrifugal force
GA	Gibberellin
GA5	Giberellic-5 acid
g_s	Stomatal conductance
h	Hour
I	Interaction
IR	Irrigation
JA	Jasmonic acid
kg	Kilogram
KPa	Kilopascal

L	Litre
m	Metre
M	Mycorrhizal inoculation
M	Molar
MDA	Malondialdehyde
mg	Milligram
min	Minute
mL	Millilitre
mm	Millimetre
mM	Millimolar
mmol	Millimole
MPa	Megapascal
MRM	Multiple reaction monitoring modes
MT	Mesophyll thickening
nm	Nanometre
nM	Nanomolar
NPQ	Non-photochemical processes
Ns	No significance
NTU	Nephelometric *Turbidity* Unit
P	Significant level
PAR	Photosynthetically active radiation
PET	Potential evapotranspiration
P_n	Net photosynthetic rate
PP	Palisade parenchyma
ppm	Parts per million
PSII	Photosystem II
psi	Pounds-force per square inch
qP	Photochemical quenching
qN	Non-photochemical quenching

RCC	Relative chlorophyll content
RH	Relative humidity
RO	Reverse osmosis
ROB	Reverse osmosis brine
rpm	Revolutions per minute
RWW	Reclaimed wastewater
RWW-	Reclaimed wastewater plants
RWW+	Inoculated Reclaimed wastewater plants
s	Second
S	Substrate
SA	Salicylic acid
SC	Scopoletin
SEM	Standard *error* of the mean
SP	Spongy parenchyma
SP	Species
T_l	Leaf temperature
TBA	Thiobarbituric acid
TBARS	Substances that react with thiobarbituric acid
tZ	Trans-zeatin
tZ-Glc	Trans-zeatin glucoside
tZ-Rib	Trans-zeatin riboside
UHPLC	Ultra-high-performance liquid chromatography
UHPLC-ESI-QqQ-MS/MS	Triple quadrupole mass spectrometry
v	Volume
V	Volt
VPD	Vapour pressure deficit
W	Watt
WS	Water-stressed conditions
WS	Water-stressed plants

WSM	Inoculated water-stressed plants
WUE	Water use efficiency
WUE$_i$	Intrinsic water use efficiency
WW	Well-watered conditions
WWTP	Wastewater treatment plant

Y (II)	Quantum yield
Y (NPQ)	Non-photochemical quantum yield

Symbols

Ψ_l	Leaf water potential
Ψ_p	Turgor potential
Ψ_r	Soil water potential at the soil-root interface
Ψ_s/Ψ_{os}	Osmotic water potential
$\Delta\Psi_s$	Differences between the initial and the end of water stress period (Ψ_s)
$\Delta\Psi_{SS}$	Contribution of dehydration
Ψ_{100s}	Osmotic water potential at full turgor
$\Delta\Psi_{100s}$	Differences between the initial and the end of water stress period (Ψ_{100s})
µL	Microlitre
µm	Micrometre
µmol	Micromoles
%	Percentage
ºC	Degree Celsius