Do Halotolerant Mycorrhizae Contribute to Halophyte *Limonium* species Growth in Saline Conditions?

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ABSTRACT

Soil salinization is an important global issue with harmful consequences for ecosystems and agriculture. A salinity problem exists if salt accumulates in the crop root zone to a concentration that causes a loss in yield. Sustainable approaches for salt-affected areas include cultivation of halophyte species and application of microbial technologies such as inoculation with halotolerant arbuscular mycorrhizal fungi that promote plant growth and development and salt tolerance. To explore the possibility of cultivating halophyte *Limonium* species (sea lavenders) in saline conditions at 200 mM NaCl, along with plants inoculation with autochthonous symbiotic fungi from a reference saline ecosystem (e.g., a saltmarsh), an experimental assay was established under greenhouse conditions. Through several physiological parameters, in non-inoculated and inoculated plants irrigated with tap and saline water, plant performance and vegetative and reproductive growth were monitored during one year. Although saline water irrigation have been positive in the beginning of the experiment and led to an extended flowering duration, continued saline exposure resulted in an impaired plant growth. An isohydric response was described for *Limonium daveaui* and *Limonium algarvense* in saline conditions. Regarding microbial inoculation, although significant effects were only observed in both *Limonium* species on some monitoring dates, positive results in plant performance were evident. Inoculated plants had higher stomatal conductance, improved thermoregulation ability, consumed more water and potentially had higher photosynthetic rates than non-inoculated plants. The enhanced reproductive development in inoculated plants, with higher flower production and seed percentage, was also revealed. During the experiment, negative effects of mycorrhizae inoculation were never detected. These findings support the successful cultivation of halophyte *Limonium* species under saline conditions while applying sustainable microbial technologies. This study raises knowledge for future cultivation of sea lavenders with several purposes contributing to salt-affected land management.

Keywords: salinity; arbuscular mycorrhizal fungi; sea lavenders; physiological status; plant reproduction.

RESUMO

A salinização antrópica do solo é uma preocupação a nível global com consequências prejudiciais para os ecossistemas e para a agricultura. Um problema de salinidade existe quando elevadas concentrações de sais solúveis se acumulam na rizosfera das culturas causando perda de produtividade. O cultivo de espécies halófilas e o uso de tecnologias microbianas são alternativas sustentáveis em áreas afetadas pela salinização. Para testar o cultivo de espécies de *Limonium* halófilas (lavandas-do-mar) em condições salinas de 200 mM de NaCl e os efeitos da inoculação com micorrizas autóctones de um ecossistema salino, foi estabelecido um ensaio experimental em estufa. Recorrendo a parâmetros fisiológicos, foi monitorizado o crescimento e o desempenho das plantas, inoculadas ou não e regadas com água da torneira e água salina, durante um ano. Embora no início do ensaio a água salina tenha potenciado efeitos positivos e prolongado a duração da floração, a exposição prolongada diminuiu o crescimento vegetativo e reprodutivo das plantas. Neste estudo, descreve-se o comportamento iso-hídrico de *Limonium daveaui* e *Limonium algarvense* em condições salinas. Relativamente à inoculação com micorrizas halotolerantes, embora os efeitos não tenham sido significativos para ambas as espécies em todas as datas consideradas, os resultados positivos no desenvolvimento das plantas foram evidentes. As plantas inoculadas apresentaram valores superiores de condutância estomática, melhoria na capacidade de termorregulação, consumiram mais água e potencialmente exibiram taxas fotossintéticas mais elevadas. O desenvolvimento reprodutivo foi também beneficiado nestas plantas, originando mais flores e sementes. Durante o ensaio não foram detetados efeitos negativos da relação simbiótica. Estes resultados suportam a possibilidade de cultivar plantas de *Limonium* halófilas em condições salinas e a aplicação de tecnologias microbianas que promovam o crescimento das culturas e a tolerância à salinidade. Realça-se o cultivo futuro de lavandas-do-mar com diversas aplicações, contribuindo para o uso sustentável e conservação de solos afetados pela salinização.

Palavras-chave: salinidade; fungos micorrízicos arbusculares; lavandas-do-mar; estado fisiológico; reprodução.

RESUMO (versão longa)

A salinização de origem antrópica é um processo de degradação do solo que pode conduzir à desertificação. Esta é uma questão relevante a nível global com consequências prejudiciais para os ecossistemas e para a agricultura, potencialmente agravada pelas alterações climáticas. Um problema de salinidade existe quando elevadas concentrações de sais solúveis se acumulam na rizosfera das culturas causando perda de produtividade. Os fatores de origem antrópica envolvidos no aumento da salinização dos solos incluem os resultantes de práticas agrícolas incorretas recorrendo ao uso de água de má qualidade (ex.: água salobra) e a práticas de rega e de drenagem inadequadas. De forma a mitigar a desertificação das áreas afetadas pela salinidade e a fazer uso desses terrenos, têm vindo a ser consideradas alternativas sustentáveis. Por exemplo, o uso de espécies de plantas halófilas que toleram elevadas concentrações de salinidade, de mais de 200 mM de NaCl, é uma alternativa para ambientes em que a maioria das culturas convencionais, por serem glicófitas, são altamente sensíveis e não conseguem sobreviver.

O género *Limonium* Mill. (lavandas-do-mar ou alfazemas-do-mar) inclui espécies de plantas halófilas que habitam zonas costeiras e estuarinas expostas a fatores ambientais, como a seca e a elevada radiação UV, concomitantes com a elevada salinidade. A espécie *Limonium daveaui* é considerada um parente silvestre de plantas cultivadas para a agricultura e nativa de Portugal Continental, com estatuto de conservação na categoria Criticamente Ameaçada. Por outro lado, *Limonium algarvense* é uma espécie que apresenta grande potencial tendo múltiplas aplicações biotecnológicas devido ao elevado teor de compostos antioxidantes, nomeadamente ao nível das suas flores, além de ser uma espécie com estatuto de conservação na categoria Quase Ameaçada. Ambas as espécies de *Limonium* estão incluídas na Lista Vermelha da Flora Vascular de Portugal Continental. Além de serem um importante recurso a conservar, possuem várias aplicações, nomeadamente, ao nível ornamental, como fonte de antioxidantes naturais, na remediação de solos contaminados e podendo ser cultivadas recorrendo a água e a substratos salinos. Das diversas características que permitem a sobrevivência destas espécies em ambientes extremos destaca-se a presença de glândulas salinas.

Para além do cultivo de espécies vegetais tolerantes à elevada salinidade, a inoculação com microrganismos mutualistas halotolerantes é uma tecnologia que pode ser usada tanto em plantas halófilas como em plantas sensíveis à salinidade. A inoculação com fungos micorrízicos arbusculares (ex.: espécies de *Glomus*) provenientes de ambientes naturalmente salinos, por exemplo sapais aos quais estão adaptados, poderá conferir diversos benefícios, promovendo o crescimento das culturas e a tolerância ao sal.

São necessários estudos adicionais para compreender quais os microrganismos halotolerantes que podem ser aplicados, quais as vantagens do seu uso, em alternativa à inoculação com microrganismos não nativos de ecossistemas salinos, e para aprofundar o conhecimento sobre as relações microrganismo-planta. Importa também compreender quais as plantas halófilas que toleram maiores concentrações de sal e que terão maior probabilidade de sobreviver em ambientes extremos. Estas plantas halófilas apresentam elevado potencial para cultivo em solos naturalmente salinos ou solos afetados pelo salinização devido a atividades humanas, e são potenciais fontes de microrganismos halotolerantes.

Para testar o cultivo de espécies de *Limonium* halófilas (lavandas-do-mar) em condições salinas de 200 mM de NaCl e os efeitos da inoculação com um consórcio de micorrizas isoladas da rizosfera de espécies de *Limonium* estuarinas, foi estabelecido um ensaio experimental com um total de quarenta plantas de *L. daveaui e L. algarvense*. Assim, estabeleceram-se quatro tratamentos experimentais, cada um com dez plantas (cinco plantas de cada uma das espécies de *Limonium* estudadas): não inoculação e rega com água da torneira; inoculação com micorrizas e rega com água da torneira; não inoculação e rega com água salina (200 mM NaCl); inoculação com micorrizas e rega com água salina (200 mM NaCl). Estas plantas cresceram em vasos, contendo uma mistura de turfa e perlite, e foram mantidas em condições de estufa durante todo o ensaio (um ano). Recorrendo a vários parâmetros fisiológicos e avaliações periódicas, foram monitorizados o desempenho das plantas e o seu crescimento vegetativo e reprodutivo.

No presente estudo, todas as plantas completaram o seu desenvolvimento, podendo ser cultivadas recorrendo a ambos os tipos de água de rega. No início do ensaio, as plantas em condições salinas apresentaram indícios de um melhor desempenho, observando-se valores de índices de refletância (NDVI e PRI) e de condutância estomática superiores aos medidos em plantas em condições não salinas. No entanto, estes efeitos positivos tenderam a desaparecer no decorrer do ensaio. Após um ano, o possível stresse causado pela exposição prolongada ao elevado nível de salinidade, evidenciado pela presença cumulativa de cristais de sal no substrato, culminou na diminuição da condutância estomática e no aumento da temperatura das folhas. Assim, descreveu-se pela primeira vez o comportamento iso-hídrico de plantas de *L. daveaui* e *L. algarvense* cultivadas em condições salinas. Os valores do NDVI e do PRI mantiveram-se semelhantes independentemente das condições de cultivo, não indiciando alterações na capacidade fotossintética. O crescimento vegetativo e reprodutivo das plantas expostas à salinidade foi também negativamente afetado pela exposição prolongada à salinidade. Nestas condições, a presença de cristais de sal na superfície das folhas evidenciou a capacidade das plantas para excretar o excesso de sais através de glândulas salinas. Apesar da diminuição do número de flores e sementes, a rega com água salina prolongou a duração do período de floração das plantas, que desenvolveram e mantiveram inflorescências durante mais tempo, ultrapassando o período previamente estudado e descrito na fenologia da floração de cada uma das espécies. Em condições não salinas, as plantas apresentaram folhas com dimensões superiores, mais flores e sementes.

Relativamente à inoculação com o consórcio de fungos micorrízicos arbusculares, foram observadas diferenças nos parâmetros analisados para as plantas nas diferentes condições experimentais, variando também consoante a data da medição e com as diferenças ambientais associadas. Os efeitos positivos da inoculação foram evidentes embora menos pronunciados em plantas cultivadas em condições salinas de 200 mM de NaCl. Os parâmetros fisiológicos estudados demonstraram um aumento tendencial dos valores da condutância estomática, da temperatura das folhas e dos índices de refletância (NDVI e PRI) nas plantas inoculadas. A capacidade de termorregulação das plantas inoculadas foi também beneficiada, mantendo temperaturas foliares mais elevadas inclusive nos meses mais frios com temperatura ambiente baixa. Além disso, a simbiose com micorrizas incrementou o consumo de água pelas plantas, que mantiveram os estomas abertos e potencialmente taxas fotossintéticas superiores. Estes efeitos podem estar relacionados com o possível aumento do sistema radicular, conferido pelas micorrizas, permitindo uma absorção de água e nutrientes mais eficiente e consequente melhoria do estado fisiológico. No final do ensaio, possivelmente devido ao crescimento prolongado em condições salinas com a acumulação contínua de NaCl, a inoculação não teve efeito nos parâmetros medidos.

A inoculação com micorrizas favoreceu o desenvolvimento reprodutivo de ambas as espécies, beneficiando características como a produção de flores e de sementes, comparativamente com plantas não inoculadas e mantidas nas mesmas condições experimentais. Este resultado demonstra a possível vantagem da inoculação das plantas com micorrizas numa perspetiva de cultivo para fins ornamentais e farmacêuticos e para reintrodução das plantas no seu habitat natural, sendo o aumento do número de flores e sementes particularmente benéfico. Também foram observadas diferenças no comportamento das espécies: embora *L. daveaui* produza maior número de flores, a percentagem de sementes (número de sementes/flores*100) foi superior em *L. algarvense*, indicando que características inerentes às próprias espécies parecem estar envolvidas, nomeadamente o nível de fertilidade das flores. Durante todo o ensaio não foram detetados efeitos negativos resultantes da relação simbiótica demonstrando a possibilidade de cultivar plantas de *Limonium* inoculadas com microrganismos halotolerantes e mantidas em condições salinas durante pelo menos um ano.

Concluindo, o uso de micorrizas nativas de um ecossistema salino (sapal) provou ser uma tecnologia microbiana importante conferindo diversos benefícios às plantas inoculadas. Assim, perspetiva-se a inoculação de outras espécies de plantas com o presente consórcio de micorrizas halotolerantes, de forma a comprovar os seus efeitos positivos, nomeadamente em culturas sensíveis ao sal, e contribuindo para o cultivo agrícola em solos afetados pelo sal.

Relativamente às espécies de *Limonium*, nomeadamente *L. algarvense*, além de tolerarem condições de salinidade muito elevadas, estas plantas podem representar uma alternativa sustentável para extração de sais do substrato devido à presença de glândulas salinas na epiderme das suas folhas. Assim, sugere-se o seu cultivo futuro em solos afetados pela salinidade, nomeadamente em esquemas de rotação de culturas ou “intercropping”. Serão necessários estudos posteriores para compreender a influência dos diversos fatores ambientais no desenvolvimento das plantas e na interação com as comunidades microbianas do solo, nomeadamente em ensaios de maior dimensão como mesocosmos.

As espécies de *Limonium* estudadas podem ser cultivadas a longo prazo em condições de estufa utilizando rega com água de elevada salinidade (200 mM de NaCl). Este ensaio permitiu estabelecer bases e contribuir para ensaios posteriores de reintrodução destas espécies nos seus habitats naturais, recorrendo a pré-tratamentos de salinidade (“priming”), inoculação com microrganismos nativos e translocação das plantas em estudo. Simultaneamente, contribuindo para a conservação de ecossistemas costeiros (incluindo os sapais) de elevado valor, atualmente em declínio e sujeitos a modificações resultantes de fatores de origem humana e agravadas pelas alterações climáticas.

Por fim, realça-se a importância de preservar estes recursos genéticos, fundamentais para o estudo e aumento do conhecimento sobre os mecanismos envolvidos na expressão de características interessantes do ponto de vista agrícola, como a tolerância à salinidade e a apomixia (produção clonal de sementes).

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AMF – Arbuscular mycorrhizal fungi

CWR – Crop wild relatives

FAO – Food and Agriculture Organization of the United Nations

g_s - Stomatal conductance

INOC – Inoculated

NDVI – Normalized difference vegetation index

NON-INOC – non-inoculated

PRI - Photochemical reflectance index

ROS – Reactive oxygen species

UV – Ultraviolet

WC - Water consumption

1. Introduction

1.1. Salt-affected Soils

Saline environments are widespread and varied, including coastal environments, saltmarshes, salt lakes and many arid areas (Bromham, 2015). Thus, salinization can be due to natural processes (primary salinization), resulting from the mineralogical and chemical characteristics of the parent materials, topography, climate, chemical composition of the groundwater, sea/tidal water level, windblown salt particles, and flood/runoff from salt-affected areas (Schofield and Kirkby, 2003; Stavi *et al*., 2021). Natural saline ecosystems, such as saltmarshes, are highly productive and valuable habitats, providing key ecosystem services, including water filtration, carbon storage and sequestration, and serving as biodiversity reservoirs (Farrer et al., 2022; Birnbaum and Trevathan-Tackett).

However, soil salinization can also result from anthropic actions (secondary salinization) which increase the concentration of readily soluble salts in the soil circulating water, containing Cl, SO_4, CO_3, HCO_3, NO_3, Ca, Mg, Na and K (Stavi *et al*., 2021; Navarro-Torre *et al*., 2023a). This phenomenon leads to severe effects on soil quality, through changes in soil structure, chemistry (Figure 1A) and biology (Morton *et al*., 2019; Hassani *et al*., 2021; Nogales *et al*., 2023). Human-inducers as unsustainable soil management, improper irrigation practices (without proper leaching and drainage) or poor-quality water use (e.g., brackish or saline water), deforestation and deep-rooted vegetation loss, improper use of fertilization through high application rate and/or amendments (e.g., manure, gypsum, or elemental sulfur) are all factors impacting soil salinization and possible desertification (Gonçalves *et al*., 2015; Stavi *et al*., 2021; Hassani *et al*., 2021; FAO, 2022). The surface or sub-surface seawater intrusion into coastal aquifers resulting from sea level rise or over-exploitation of fresh underground water is also associated with soil salinization (Hassani *et al*., 2021; FAO, 2022). Soil salinization due to human activities represents a global challenge being currently one of the main causes of soil degradation, putting ecosystems environmental sustainability and agricultural productivity at risk (Stavi *et al*., 2021; Navarro-Torre *et al*., 2023a). The extent of salt-affected land is increasing rapidly, with an estimated 24 million hectares of salt-affected soils in Europe and 1171.8 Mha worldwide, exacerbated by climate change (Hassani *et al*., 2020). Forecasted increasing temperatures and growing frequency and intensity of droughts, alongside with the aggravated drying and expansion of the world's drylands, increases both soil and water salinization processes (Cook *et al*., 2014; Bromham, 2015; Stavi *et al*., 2021; Hassani *et al*., 2021).

The Mediterranean coastline is considered the European “hotspot” of soil salinization, mainly resulting from human activities affecting 25% of irrigated agricultural land (Daliakopoulos *et al.*, 2016; Navarro-Torre *et al.*, 2023a). In Portugal, the main affected areas are western and south coastal regions as well as irrigated agricultural inland areas in the southern region with semi-arid to arid climates. In 2015, it was estimated that around 150,000 ha were salt-affected, among which 50,000 ha due to anthropic causes such as bad quality water uses and inadequate irrigation and drainage practices (Gonçalves *et al.*, 2015). The increase of the irrigated area and the prospects of climate change for the incoming decades, including rising temperatures, can lead to an increase of the degraded area in Portugal and in other Mediterranean countries (Gonçalves *et al.*, 2015). Thus, there’s an increase interest in sustainable alternatives to use these lands as well as to mitigate the salinization problem (Stavi *et al.*, 2021; FAO, 2022; Navarro-Torre *et al.*, 2023a).

1.2. The Response of Plants to Salt Stress – Saline Agriculture

Salt stress is one of the most dangerous threats for crop production limiting globally the agricultural productivity since food production relies mostly on salt sensitive species (glycophytes) (Flowers and Flowers, 2005; Flowers and Colmer, 2008; Estrada *et al.*, 2013; Navarro-Torre *et al.*, 2023a). Salinity presents challenges for plant growth through ions toxicity, interference with the uptake of essential nutrients, lowering the water potential and increasing osmotic stress (Ayers and Westcot, 1985; Flowers and Flowers, 2005; Bromham, 2015; Morton *et al.*, 2019; Stavi *et al.*, 2021). The high amounts of water-soluble salts in the soil increases the osmotic potential, hence reducing the water potential between soil solution and plant root cells and therefore water uptake by the roots slows down causing drought stress (Ayers and Westcot, 1985; Flowers and Flowers, 2005; Stavi *et al.*, 2021; Morton *et al.*, 2019). Salinity stress affects photosynthesis, respiration and distribution of photoassimilates causing plant growth inhibition, wilting, drying and death of entire organs of most plants and vegetable crops. If grown under saline conditions glycophytes suffer a decline of several physiological parameters and yield (Zörb *et al.*, 2019; Navarro-Torre *et al.*, 2023a) but some cultivars are more tolerant than others to moderate saline conditions (4-8 dS/m) (Navarro-Torre *et al.*, 2023a). For instances, Mediterranean crops such as *Ficus carica*, *Punica granatum*, *Phoenix dactylifera* and several ornamental plants are considered moderately salt tolerant species, while certain staple foods such as sugar beet, sugarcane and vegetables like radish present some genotypes tolerant to salinity (Ayers and Westcot, 1985; Navarro-Torre *et al.*, 2023a). Furthermore, halophyte species are salt tolerant plants that survive to reproduce in environments where the salt concentration is around 200 mM

NaCl or more (Flowers and Colmer, 2008). These plants have evolved traits that allow them to persist, grow rapidly and complete their life cycles in saline harsh environments (Figure 1B) (Flowers and Colmer, 2008; Bromham, 2015).

Figure 1 – Example of salt-affected soils with solid phases (efflorescences), usually with high solubility, on the soil surface. (**A**) The dense salt crusts of solid phases from these soils can crack in dry conditions whereas (**B**) in wet conditions these may not fully dissolve as in Tagus and Guadiana estuaries (southwest Europe, Portugal and Spain). Halophytes, as *Salicornia ramosissima* J. Woods and *Sarcocornia* sp. have high salinity tolerance and can grow naturally in salt-affected soils from lower saltmarshes (adapted from Navarro-Torre *et al*., 2023a).

Salt-affected soils can be used for halophytes cultivation, since these species can grow and reproduce in saline conditions where conventional crops cannot. Some of these species can be cultivated using brackish or saline water or saline aquaculture effluents as irrigation and fertilizers for plant production as well as in saline sediments and using soil technologies (e.g., Technosols construction) (Rodrigues *et al*., 2020; Cortinhas *et al*., 2020, 2021; Cayenne *et al*., 2022; Nogales *et al*., 2023; Navarro-Torre *et al*., 2023a). Halophytes species have several uses related to their applications as fodder, forage, grazing, ornamental, and landscape plants, bioremediation purposes, phytostabilisation and rehabilitation of contaminated water (e.g., recycling hyper-saline drainage water for later use in agriculture) (Flowers and Colmer, 2015; Santos *et al*., 2016; Cortinhas *et al*., 2019). These plants have potential applications on pharmaceutical industry due to metabolites they produce as response to salinity. Additionally, they can be considered potential foods and used as nutraceuticals and dietary supplements, important for the agro-food industry, and have been widely used in traditional medicine (Flowers and Colmer, 2015; Rodrigues *et al*., 2015, 2016, 2020; Cortinhas *et al*., 2019). Besides being considered an important reservoir of bioactive molecules with multiple biotechnological applications, halophytes can even be used as sources of biofuel or in glass industry (Cortinhas *et al*., 2019). Due to their ability to uptake significant amounts of elements associated to salinity, they can be used as NaCl removing crops, as an intercrop, or included in rotation programs as an environmentally friendly approach (Hamed *et al*., 2021). Halophytes may also be useful as means to engineer salt tolerance in model species and ultimately in conventional crops, both in terms of understanding target traits and as genetic resources (Flowers and Flowers, 2005; Flowers and Colmer, 2008, 2015; Dassanayake and Larkin, 2017; Morton *et al*., 2019).

1.3. Salt Tolerance Mechanisms in Halophytes

Halophytes are relatively uncommon among angiosperms and tolerate salt concentrations that kill 99% of the other species, representing *circa* 1% of the world's flora (Flowers and Colmer, 2008). These plant species inhabit different types of saline habitats, both humid and arid, such as near-shore shallows and estuaries, land within the tidal zone (e.g., mangrove forests), littoral and inland saltmarshes, dunes, cliffs by the sea, salt lakes and salt deserts (Flowers and Colmer, 2015; Hassan *et al*., 2017). To adapt to salt stress in their environment, halophytes have developed various mechanisms or combinations of traits at the biochemical, physiological, anatomical, and morphological levels (Flowers and Flowers, 2005; Flowers and Colmer, 2008; Bromham, 2015; Grigore and Toma, 2017; Acosta-Motos *et al*., 2017). Among such adaptations, these species produce and accumulate metabolically compatible organic solutes (osmolytes; e.g., proline) in the cytoplasm to balance the osmotic potential of Na^+, K^+ and Cl^- compartmentalized into vacuoles through a variety of transporters (Flowers and Colmer, 2008, 2015; Hassan *et al*., 2017; Acosta-Motos *et al*., 2017; González-Orenga *et al*., 2021). The accumulation of osmolytes can protect macromolecular structures and avoid reactive oxygen species (ROS) toxicity, which can disrupt the integrity of cellular membranes and the enzymatic activity (Hassan *et al*., 2017; González-Orenga *et al*., 2021). To avoid ROS toxicity, plants produce antioxidant enzymes, for example, catalase and superoxide dismutase, and non-enzymatic antioxidants such as ascorbic acid, glutathione, flavonoids and other polyphenolic compounds (Flowers and Colmer, 2008; Hassan *et al*., 2017; González-Orenga *et al*., 2021). Another adaptation is the use of different mechanisms for salt exclusion, salt accumulation or salt excretion (Flowers and Colmer, 2008; Bromham, 2015). Particularly, some halophytes have salt glands in both leaves and stems that compartmentalize and excrete toxic ions to the outside of the plant (Flowers and Colmer, 2005; Flowers and Colmer, 2008; Dassanayake and Larkin, 2017; Caperta *et al*., 2020). Succulence is also a trait present in some halophytes reflecting an increase in leaf thickness due to an increase in cell size and a high water content per surface area unit (Flowers and Flowers, 2005; Flowers and Colmer, 2015; Acosta-Motos *et al*., 2017). Traits associated with reproductive development include increase flower number, decreased sterility and seed heteromorphisms and in some species high seed quality under salinity conditions (Yuan *et al*., 2019). Finally, life history traits can also play a role in salt tolerance like dormancy during dry periods when soil salinity is highest by restricting seed germination to a favourable time, increasing the chance of seedling survival (Cao *et al*., 2014). Seeds remain non-germinated until precipitation event(s), which increase water content and decrease soil salinity, sufficient for seed germination and seedling establishment (Cao *et al*., 2014).

1.4. Microbial Symbiotic Interactions Increase Plants' Salt Tolerance - Arbuscular Mycorrhizal Fungi

Plants interact with diverse taxonomically structured communities of microorganisms that inhabit in the soil, rhizosphere, roots and other plant tissues and establish complex and dynamic interactions with the host plant (van der Heijden *et al*., 2015; Zahir *et al*., 2019; Trivedi *et al*., 2022). In exchange for carbon compounds, these microorganisms play a crucial role in promoting plant growth and development, photosynthesis, nutrient uptake and phytohormones production (e.g., auxins, gibberellins and abscisic acid) as well as in promoting plants tolerance to biotic and abiotic stresses, including salinity (Figure 2) (Porcel *et al*., 2012; Kapoor *et al*., 2013; Zahir *et al*., 2019; Zhu *et al*., 2022). A remarkable survival strategy adopted by both glycophytes and halophytes to survive in saline environments is the association with different halotolerant soil microorganisms (Zahir *et al*., 2019; Pan *et al*., 2020; Trivedi *et al*., 2022). These microorganisms have evolved series of mechanisms to adapt to different salinity conditions in a wide range of saline environments (Porcel *et al*., 2012; Kapoor *et al*., 2013; van der Heijden *et al*., 2015; Zahir *et al*., 2019; Trivedi *et al*., 2022). Thus, when interacting with host species they contribute to a faster plant adaptation to saline soils, potentially, a less intense transplantation shock, and a faster adaptation and recovery after salt stress exposure, stimulating plant re-growth (Kapoor *et al*., 2008; Trivedi *et al*., 2022; Navarro-Torre *et al*., 2023a,b).

Among these halotolerant microorganisms are arbuscular mycorrhizal fungi (AMF) belonging to the Glomeromycota, which are obligate biotrophs, forming mutualistic symbiosis with *circa* 74 % of plant species (Kapoor *et al*., 2013; van der Heijden *et al*., 2015). Although the symbiotic association between AMF and their hosts is considered as non-specific, host preferences and host selectivity have been reported, existing physiological or morphological differences within species and even geographic or ecotypic fungi isolates (Tian *et al*., 2004; Estrada *et al*., 2013; van der Heijden *et al*., 2015). Arbuscular mycorrhizal fungi constitute an integral component of the natural ecosystem and widely exist in saline environments like saline inlands and coasts and saltmarshes (Tian *et al*., 2004; Estrada *et al*., 2013; Zahir *et al*., 2019). The use of salinity adapted AMF ecotypes should be rewarding (Estrada *et al*., 2013) as they help several host plants to cope with a variety of severe salinity stress conditions, which present additional concomitant stress factors as high UV radiation, low water availability or waterlogging (Tian *et al*., 2004; Porcel *et al*., 2012; Estrada *et al*., 2013; Navarro-Torre *et al*., 2023a). These fungi are important drivers of global carbon and nutrient cycles, have a large impact on seedling establishment, litter decomposition, soil formation and aggregation, besides improving rooting and plant establishment and enhancing plant community diversity (Hammer and Rillig, 2011; Porcel *et al*., 2012; Kapoor *et al*., 2013; van der Heijden *et al*., 2015).

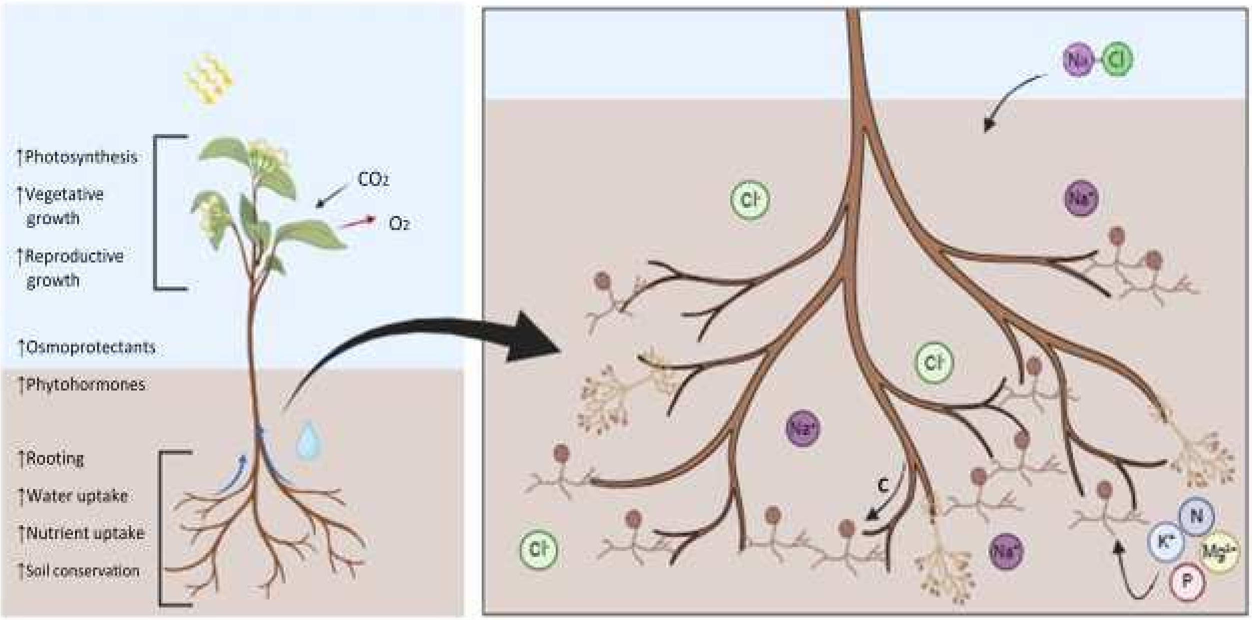


Figure 2 – Schematic representation of several microbial benefits to plants under saline conditions. The association with halotolerant soil microorganisms, such as *Glomus* spp., is a survival strategy adopted by both glycophytes and halophytes. In exchange for carbon compounds, mutualistic microorganisms promote plant growth and development, photosynthesis, nutrient uptake, plant-water status and phytohormones production**.** Halotolerant microorganisms enhance plant tolerance to saline stress by preventing element toxicity through ion entry restriction, exclusion or immobilization in different plant, soil, or fungal compartments, by excretion of glomalin-related proteins and accumulation of osmoprotectant compounds.

Microbial based techonologies can be employed as a sustainable strategy to improve the growth and reproduction of both glycophytes and halophyte species in salt-effected soils (Zahir *et al*., 2019; Navarro-Torre *et al*., 2023a; Nogales *et al*., 2023). The use of halotolerant microbes as biopesticides and biofertilizers could be a cost-effective and environment friendly approach in agricultural system, potentially decreasing reliance on synthetic agrochemicals (Zahir *et al*., 2019). Although salt stress can affect negatively AMF by inhibiting mycelial growth and spores germination at certain salt concentrations (Zahir *et al*., 2019; Zhu *et al*., 2022), many studies reported higher salt tolerance and improved growth (greater number of leaves, increased shoot and root dry biomass) and performance of mycorrhizal plants than non-inoculated ones under salt stress conditions (Porcel *et al*., 2012; Kapoor *et al*., 2013; Zahir *et al*., 2019; Pan *et al*., 2020; Nogales *et al*., 2023). Additionally, AMF may promote higher reproductive development, for instances, by advancing flowering time, increasing flower size, the amount of pollen, seed number, biomass and germination percentage (Zhu *et al*., 2022). These positive effects are explained by a higher root development that can explore more soil volume in search of water and nutrients that are limiting to plant growth, especially nitrates and phosphates, improving water use efficiencies, plant–water status and plant nutrition (higher K^+ /Na^+ ratios in plant tissues) (Porcel *et al*., 2012; Estrada *et al*., 2013; Kapoor *et al*., 2013; van der Heijden *et al*., 2015; Pan *et al*., 2020). The efficient nutrient uptake contributes to leaching losses reduction of both organic and inorganic nutrients (van der Heijden *et al*., 2015). Arbuscular mycorrhizal fungi also enhance photosynthetic capacity and efficiency, enhancing chlorophyll and carotenoid synthesis (preventing it's degradation), stomatal conductance, transpiration and photosynthetic rates (Porcel *et al*., 2012; Kapoor *et al*., 2013; Zahir *et al*., 2019; Pan *et al*., 2020; Zhu *et al*., 2022; Navarro-Torre *et al*., 2023a). Besides, the presence of different concentrations and distributions of Na^+ and Cl^- within different fungal and plant tissues indicates that AMF can prevent element toxicity by ion entry restriction, exclusion or immobilization in different plant, soil or fungal compartments, and by the excretion of glomalin-related proteins (Porcel *et al*., 2012; Estrada *et al*., 2013; Kapoor *et al*., 2013; Navarro-Torre *et al*., 2023a). These macromolecules are stable hydrophobic glycoproteins that play an important role in soil structure stabilization and contribute to soil conservation (Hammer and Rillig, 2011; Kapoor *et al*., 2013). A better osmotic adjustment is also achieved by osmoprotectant compounds accumulation, such as proline, glycine, betaine and soluble sugars that play roles in scavenging free radicals, stabilizing sub-cellular structures and buffering cellular redox potential under stress conditions (Tian *et al*., 2004; Porcel *et al*., 2012; Pan *et al*., 2020).

The symbiosis differentially regulates the expression of several plant genes. For example, AMF have been associated to an enhanced expression of aquaporin genes in some organs of plants exposed to salt stress, possibly improving plant–water relations and reflecting an enhanced supply with phosphorus and other minerals (Ouziad *et al*., 2006; Porcel *et al*., 2012; Sharma *et al*., 2021). Furthermore, AMF enhance the activity of enzymatic (e.g., superoxide dismutase, catalase, glutathione reductase and ascorbate peroxidase) and non-enzymatic (e.g., carotenoids, ascorbic acid, glutathione, tocopherols) antioxidants in order to restrict excessive generation of ROS and lipid peroxidation, thus improving membrane integrity and permeability, protecting plant cells from oxidative damage induced by salinity (Porcel *et al*., 2012; Kapoor *et al*., 2013; Pan *et al*., 2020).

1.5. Halophyte *Limonium* species (Plumbaginaceae)

The Plumbaginaceae is a representative family of flowering plants including halophytes (Caperta *et al*., 2020; Malekmohammadi *et al*., 2024) and in which different species establish symbiotic relations with halotolerant and halophilic microorganisms in natural environments (Navarro-Torre *et al*., 2023b). This cosmopolitan family comprises herbs, shrubs or small trees, rarely climbers, adapted to a wide range of extreme environments, such as arid and saline habitats (Kubitzki, 1993). Characteristics of salt and drought tolerance are distributed through the members of this family which present adaptations involving anatomy (e.g., multicellular glands) and physiology (e.g., osmoprotective compounds and ion compartmentation) (Flowers and Colmer, 2008; Slama *et al*., 2015; Caperta *et al*., 2020). For instances, several recretohalophytes (salt excretion) evolved salt glands to secrete a range of ions (Na^+ , K^+ , Ca^{2+} , Mg^{2+} , Cl^-, HCO_3^-, SO_4^{2-}) and other elements (As, Cd, Cr, Cu, Fe, Mn, Ni, Pb and Zn) (Dassanayake and Larkin, 2017; Caperta *et al*., 2020). A complex genus belonging to the Plumbaginaceae is *Limonium* Miller, commonly known as sea lavenders, the most species-rich and widespread comprising annuals and perennials that can produce sexual and/or asexual seeds (apomixis) (D'Amato, 1940; Róis *et al*., 2016; Conceição *et al*., 2019, 2021; Hörandl *et al*., 2024).

Sea lavenders are distributed over all continents but especially in suitable coastal strips in Europe, being the largest number of species present in the Mediterranean region (Erben, 1978, 1993; Malekmohammadi *et al*., 2024). The genus includes at least 45 halophytic species that inhabit coastal areas and inland-saline and gypsum ecosystems from the arctic to the tropics, such as sea and rocky shores and cliffs sand dunes, saltmarshes (Figure 3), salt steppes, salt meadows, salt pans and shallow salt lakes (Erben, 1993; Caperta *et al*., 2020; Malekmohammadi *et al*., 2024). Many of these saline environments include priority habitats such as Mediterranean salt steppes, with soils temporarily permeated by saline water, along Mediterranean coasts and on the fringes of Iberian salt basins (European C., 2013). *Limonium* species can be an excellent source of halotolerant and halophilic microorganisms to potentially improve crop performance under saline stress conditions (Navarro-Torre *et al*., 2023b).

Figure 3 – *Limonium* species inhabiting coastal strips. (**A**) *Limonium binervosum* thriving in a coastal habitat, sea and rocky shores and cliffs (São Pedro de Moel, Leiria, Portugal; credits: Catarina G. Domingues); (**B**) *Limonium algarvense* thriving in a saltmarsh during the low tide (Algarve, Portugal; credits: Ana D. Caperta).

Sea lavenders can be considered facultative halophytes since beside the natural saline environments where found they can also grow in the absence of salinity (Erben, 1978; González-Orenga *et al*., 2021). The genus comprises many endemic, rare and threatened taxa with different conservation status, catalogued worldwide in red lists, red books, or lists of protected species at the national and regional levels (Kaninski *et al*., 2012; Carapeto *et al*., 2020). Negative anthropic activities (e.g., urbanisation of areas coinciding with native habitats, uncontrolled harvesting of cut flowers, trafficking, pollution, etc) are the main causes, seriously altering the density and size of wild populations and diminishing their regeneration potential (Kaninski *et al*., 2012; Carapeto *et al*., 2020; Cortinhas *et al*., 2021). An example of a critically endangered halophyte is the Lusitanian endemic *Limonium daveaui* Erben (Figure 4) (Caperta and Carapeto, 2020) that is included in a national list of crop wild relatives (CWR) (Brehm *et al*., 2008). *Limonium daveaui* is related to species of direct socio-economic importance as ornamental *Limonium sinuatum* and may contain target genes that can enhance desirable characteristics in crops (Brehm *et al*., 2008). With a large distribution along the west coast of Portugal in the past (Erben, 1978; Caperta and Carapeto, 2020), *L. daveaui* is currently restricted to the marshes of Tagus Estuary (SW Portugal, SW Europe) forming only few local populations with small size (Caperta and Carapeto, 2020). Among the reasons involved in the continuous decline of the species in this estuary are the deterioration of its habitat quality due to invasive species and anthropic pressures like human trampling (Caperta and Carapeto, 2020), industry activities (petrochemical and steelwork) requiring large spaces and suitable terrains, chemicals, ship construction and repair, cement manufacture, agriculture fertilizers and pesticides with consequent soil contamination (e.g., high levels of anthropic Hg) (Cortinhas *et al*., 2021). Understanding the biology and microenvironmental requirements as well as performing taxonomic and demographic studies with long-term monitoring of *Limonium* populations are required for successful cultivation and reintroduction of this and other rare species (Godefroid *et al*., 2011; Maschinski and Albrecht, 2017; Cortinhas *et al*., 2021).

Figure 4 – *Limonium daveaui* plants. (**A**) *Limonium daveaui* plant in the Tagus Estuary marshes (Fundação do Samouco salterns complex, Alcochete, Portugal; credits: Catarina G. Domingues); (**B**) *Limonium daveaui* plants cultivated under greenhouse conditions (credits: Teresa C. Ferreira).

Besides their conservation status and contribution to natural saline environments biodiversity, *Limonium* species have several other important applications. For instances, sea lavenders are well known in international cut flower markets since they present several features like multiple flower stems, coloured calices that remain open for long periods after flower senescence making them suitable as fresh and dry cut flowers that are of commercial value (Morgan *et al*., 1998, 2001; Kaninski *et al*., 2012). They are also appropriate ground cover plants for the urban landscape (Kaninski *et al*., 2012) and widely considered as potential ornamental candidates and as animal fodder for dry and saline areas in arid zones

(Mahmoudi *et al*., 2023). Their importance and cultivation for the ornamental industry promoted the creation of new varieties over many years, breeding different species and trying to incorporate traits from less well known species into new cultivars, thus making them important genetic resources (Morgan *et al*., 1998, 2001; Kaninski *et al*., 2012).

Similarly to other halophytes, *Limonium* spp. produce metabolites with diverse bioactivities, such as antioxidant, antimicrobial, cytotoxic, antifungal, antibacterial, antitumoral, antiviral and immunomodulatory (Rodrigues *et al*., 2015, 2020; Cortinhas *et al*., 2019, 2020; Mahmoudi *et al*., 2023). An interesting recretohalophyte with such characteristics is *Limonium algarvense* Erben (Figure 3B) (Rodrigues *et al*., 2015, 2020; Cortinhas *et al*., 2019, 2020; Caperta *et al*., 2020) a protected species that has been assessed as Near Threatened in Portugal (Carapeto *et al*., 2020). It is an Iberian-Moroccan (west Mediterranean) endemism found in coastal sand dunes and saltmarshes, occurring frequently on alluvium (Espírito-Santo *et al*., 2012; Caperta *et al*., 2017). Both *L. algarvense* and *L. daveaui* are listed in the Red List of Vascular Plants of mainland Portugal (Carapeto *et al*., 2020). *Limonium algarvense* presents an antioxidant defence system associated with high phenolic compound (phenolic acids, flavonoids, and tannins) content, thus, representing a promising source of natural bioactive compounds with potential applications in fields such as the agro-food industry (e.g., as a functional beverage) (Rodrigues *et al*., 2015, 2016, 2020). *Limonium algarvense* flowers' infusions and decoctions have similar or even higher antioxidant and anti-inflammatory properties than green tea, and thus, may be useful for alleviating symptoms associated with oxidative (including coronary diseases and cancer, and age-related degenerative brain disorders) and inflammatory related diseases (Rodrigues *et al*., 2016; Cortinhas *et al.,* 2019). Previous studies demonstrated that both halophytes *L. algarvense* and *L. daveaui* can be successfully cultivated in saline soils and irrigated using fresh, brackish and saline water (Cortinhas *et al*., 2020, 2021; Nogales *et al*., 2023). In *L. algarvense* plants cultivated at 300 mM NaCl the relative abundance of several compounds linked to salinity tolerance changed in comparison with extracts from plants in non-saline conditions (Rodrigues *et al*., 2020).

The need for sustainable agricultural practices is increasing given the current context of soil and water rapid degradation and scarcity in many parts of the world, the increasing demand for food and freshwater and the impacts of climate changes. Thus, research regarding halophyte species and associated halotolerant and halophilic microorganisms that can increase plant performance and stress tolerance are of great importance (Estrada *et al*., 2013; Bromham *et al*., 2015; Flowers and Colmer, 2015; Morton *et al*., 2019; Zahir *et al*., 2019; Nogales *et al*., 2023; Navarro-Torre *et al*., 2023a,b). Soil and microbial technologies can be successfully applied in saline conditions to cultivate *Limonium* spp. with agronomic and economic value while contributing to the sustainable use of marginal lands (Nogales *et* *al*., 2023). Additionally, the use of native microorganisms from saline reference ecosystems (e.g., saltmarshes) with specific adapted microbial communities can enhance AMF benefits (Tian *et al*., 2004; Estrada *et al*., 2013; Zahir *et al*., 2019).

1.6. Hypothesis and Goals

Studies are needed to identify which halotolerant microorganism species can be applied and the advantages of their use, and to deepen understanding about microorganism-plant relationships. It is also important to determine which halophyte species tolerate higher salt concentrations and are more likely to survive in extreme environments.

The hypothesis of the present study was that the inoculation with AMF, sourced from a saline reference ecosystem (e.g., a saltmarsh), could promote sea-lavenders' performance and tolerance to salinity. Particularly, the objectives of this work were to: 1) test the effect of the inoculation with a halotolerant AMF consortium in *L. algarvense* and *L. daveaui* plants exposed to saline water irrigation at 200 mM NaCl, and assess potential benefits in improving host's salinity tolerance and vegetative growth; 2) evaluate the plant reproductive growth in terms of floral phenology, inflorescences, flower and seed production in inoculated and non-inoculated plants under greenhouse conditions.

2. Materials and Methods

2.1. Study Species

In this study, we focused on *L. algarvense* and *L. daveaui*, two chamaephytes occurring on thermomediterranean dry saltmarshes. The studied *Limonium* plants have leafless and underground woody stems, with leaves usually in basal rosettes, and inflorescences in terminal panicles branched in the lower third (Erben, 1993). The seasonal phenology of these species, grown in pots with a peat and perlite mixture and maintained under controlled greenhouse conditions is shown in Figure 5 (Cortinhas *et al.*, 2020, 2021; Nogales *et al*., 2023). The vegetative growth is prominent during spring and until flowers developed. The inflorescences (scapes) present few sterile branches. The branches, consisting of spikelets of 1–5 bluish violaceous flowers with 3 bracts aggregated into spikes, bloom between March and July in *L. algarvense*, and between June and September in *L. daveaui*. Each fertile flower gives rise to a small capsule with a single seed (Erben, 1993) and, after fruit ripening, these species enter in a vegetative dormancy phase characterized by low vegetative growth.

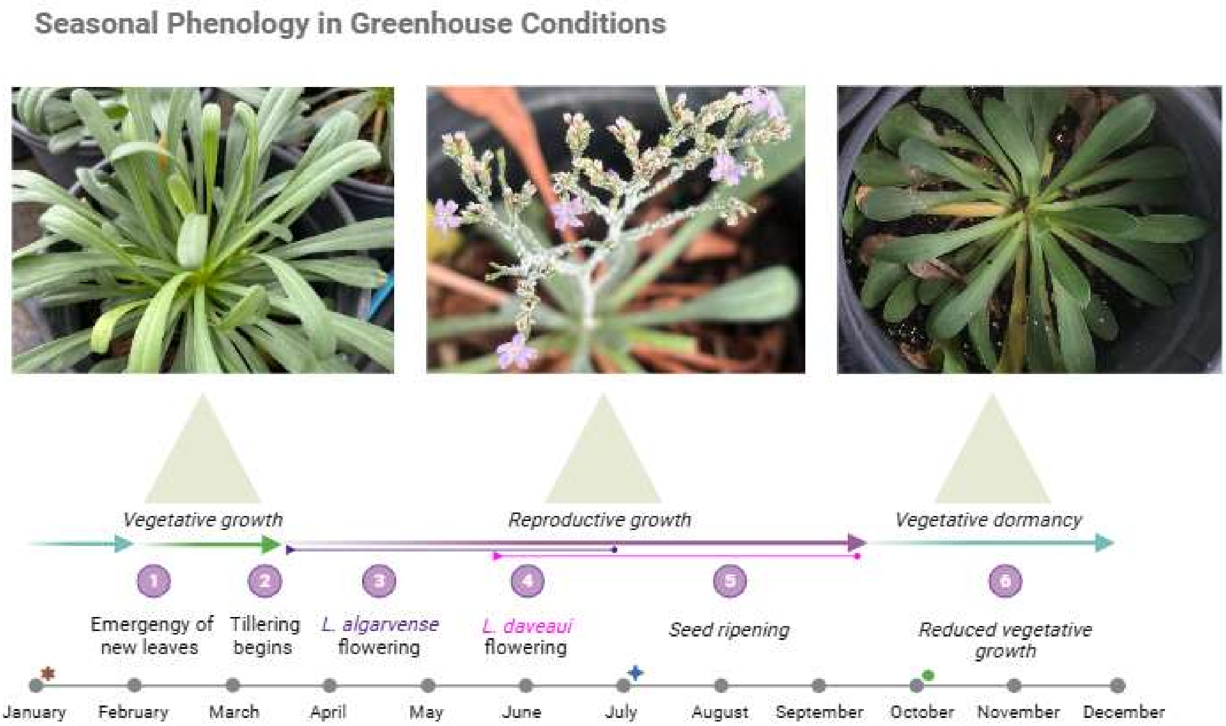


Figure 5 - Seasonal phenology of *Limonium daveaui* and *Limonium algarvense* plants grown under greenhouse conditions at Instituto Superior de Agronomia, University of Lisbon, Portugal (adapted from Cortinhas *et al*., 2020, 2021; Nogales *et al*., 2023). Different symbols in the timeline indicate the steps performed for the experiment establishment at ISA's greenhouse. (●) Seed germination in October 2022; (✱) AMF inoculation in January 2023; (✦) saline water treatment from July 2023 to July 2024.

2.2. Experimental Set Up

In this experiment twenty plants per species, in a total of forty plants, were established in a greenhouse at the Instituto Superior de Agronomia (ISA), University of Lisbon, Portugal. *Limonium algarvense* plants were originated from seeds collected from natural populations in saltmarshes in Guadiana estuary (Algarve, Portugal) and *L. daveaui* in Fundação do Samouco salterns complex (Alcochete, Portugal) and germinated as described in Róis *et al.* (2012). In brief the seeds were placed in Petri dishes with water soaked filter paper in a growth chamber (Rumed) under controlled conditions (temperature 26 °C / 22 °C with 16 h light / 8 h dark photoperiod, respectively). After three months growing in *jiffy* pots, the obtained seedlings were transplanted to 3 L round plastic pots filled with a mixture of autoclave-sterilized (1 h at 120°C) peat and perlite (1:2 v/v). At that time, in January 2023, substrate of half of the pots were inoculated with the native AMF consortium by placing a layer of inoculum (7.6 g in total - 200 spores approximately) in the middle of two substrate layers in each pot, following the procedure in Nogales *et al*. (2023). The halotolerant AMF consortium, isolated from *L. daveaui* rhizosphere, was previously analysed and 75% of its composition was *Rhizophagus invermaius* and 35% other members of Glomeromycota (Gomes-Domingues *et al*., in preparation). These steps were previously carried on and the plantlets grew under greenhouse conditions and were irrigated with tap water for five months until the symbiotic relation established and confirmed (Gomes-Domingues *et al*., in preparation). During the two vegetative phases (Figure 5), 10 g of Proteas inorganic NK fertilizer (Siro) was applied to each plant.

In July 2023, half of the inoculated and non-inoculated plants were watered with a saline solution with 200 mM sodium chloride (NaCl). The selection of 200 mM NaCl was based on prior research indicating that halophytes can survive in such salinity concentration (Flowers and Colmer 2008). Saline water irrigation was provided directly into trays by an automatic system regulated according with plants seasonal variation. This system consisted in a low capacity and suitable for saline water Eheim CompactOn 2100 electrobomb inside a 50 L water tank. Trays and electrobomb were connected by a 16 mm tube. Each tray had associated 20 self-compensating drippers. The system was regulated to ensure that the same input of water (saline or tap) was applied to the respective treatments.

From the beginning of the saline water irrigation treatment (July 2023) until the end of the experiment (12 months), five plants of each species were maintained under each of the four experimental conditions: inoculated (inoc) and non-inoculated (non-inoc) with freshwater (tap water) irrigation, and inoc and non-inoc with saline water irrigation. During all experiment, position of pots in the trays was changed approximately every 10 days so that each plant was not always in the same place. When temperatures start to increase, mainly during summer months (June, July, August and September), a shadow cover was placed to minimize the high temperatures and light intensities reached inside the greenhouse.

2.3. Plant Performance and Growth

To evaluate plants physiological status between the distinct treatments, plant performance was monitored by reflectance index assessment (Photochemical Reflectance Index, PRI, and Normalised Difference Vegetation Index, NDVI) and by foliar gas exchange parameter measurements. The PRI and NDVI were measured using PlantPen PRI 200 (Photon Systems Instruments-PSI, Czech Republic) and PlantPen NDVI 300 (PSI, Czech Republic) portable devices, respectively. The PRI is indicative of photosynthetic light use efficiency and plant stress levels. While NDVI is an indicator of chlorophyll content, phosphorus and nitrogen nutrition, related to the photosynthetic capacity and commonly used to predict biomass and plant vigor (Sembiring *et al.*, 1998; Garbulsky *et al.*, 2011; Cortinhas *et al.*, 2021). Foliar gas exchange parameter (stomatal conductance to water vapour-g_s) was recorded using a LI-600 Porometer/Fluorometer (Licor 600, LI-COR Bio Sciences, USA) to obtain high-throughput stress evaluations. All measurements were non-destructive and performed once a month always at the same time, between 12 and 14 pm, in three random leaves per plant selected from the young leaves on the plant central core. All indexes were determined for all plants under different conditions and irrigation regimes.

Leaf temperature, commonly used as an indicator of plant stress, was also measured through thermal images collection as it is an adequate non-destructive approach easily applied both in the field and in greenhouse studies (Jones 2002, 2004; Costa *et al.*, 2010). Similar to that described by Navarro-Torre *et al.* (2023b), thermal images were collected using a FLIR A35 thermal camera (Teledyne FLIR®, Germany) equipped with an infrared temperature sensor with manual focus and a spectral range between 7.5 and 14 μm, and a 320 × 256 pixel resolution. Data were collected always at 12 pm. Thermal images were acquired using FLIR Tools® v6.4 and analyzed with ImageJ® software v1.53 (National Institutes of Health, USA). Images were converted to 8 bit and calibrated so that the gray scale corresponded to the temperature range recorded by the thermal camera. To determine the average temperature of the entire plant leaf area, the selected pixels corresponding to the plant leaves were analyzed. Leaf temperature values were not normalized according to dry and wet leaf references (Jones, 2004; Costa *et al.*, 2010) since the aim of the trial was to compare differences between experimental treatments at each time point. Additionally, it was also considered the difference between room and leaf temperatures recorded at each measurement time point.

Plant growth was monitored once a month by counting the number of leaves (total green and dry leaves) per plant, regardless their size. At the end of the experiment, the number of green leaves was counted, and plants and leaves diameter were measured using a ruler, to compare plant growth between different treatments as in Brancaleoni *et al.* (2018). For leaf size measurement, three leaves per individual were used (15 leaves/treatment). In fructifying scapes with approximately four to nine weeks, flowers and mature seeds were harvested and counted. The proportion between number of seeds and dried flowers was calculated considering the number of Isolated Seeds/Flowers*100 (%). All scapes were covered with suitable caps in the beginning of flowering season to isolate flowers and prevent possible crosses with pollen from different individuals.

After saline water irrigation, the mean water consumed monthly per plant at each experimental condition was estimated considering Water Consumption (WC) = Input Water -

Non-Absorbed Water. The non-absorbed water was measured weekly using the water remaining in trays after freshwater or saline water irrigation and considering the seasonal water input variation. Since all plants were maintained in equal greenhouse conditions and once again the aim of the trial was to compare differences between experimental treatments, other external factors such as water evaporation were not included in the equation.

2.4. Statistical Analysis

Five dates of data collection were selected considering plant seasonal phenology in greenhouse conditions (Figure 5) as well as AMF symbiotic relation establishment, the timings of saline water irrigation and subsequent plant effects. Due to missing values in data collection, it was not possible to analyse g_s values for all the chosen dates. The data collected for the two *Limonium* spp. were considered independently. Values gathered in July 2023 were analysed for each parameter by a one-way ANOVA with AMF inoculation as main factor, since it was when saline water irrigation started. For the other selected dates, data for the different experimental treatments were analysed by a two-way ANOVA with interaction where AMF inoculation and irrigation treatments were considered as main factors. Moreover, since differences in g_s values were observed between species by the end of the experiment, a two-way ANOVA was also performed for each irrigation regime, with species and AMF inoculation as main factors. The total number of leaves recorded between September 2023 and June 2024 for each species was statistically analyzed using a linear mixed model, with the factors time, AMF inoculation, and irrigation. Prior to analysis, a square root transformation was applied to leaf number data to normalize the distribution. For all parameters, when the data were statistically significant ($p < 0.05$), pairwise-comparisons were carried out using a Tukey HSD post hoc test. In all cases data normality and variance homogeneity were tested prior to the analyses. Statistical analyses were performed using statistical software R Studio version 4.4.0 (2024-04-24 ucrt) for Windows.

3. Results

3.1. Plant Physiological Responses to AMF Inoculation and Saline Water Irrigation

Overall, there was a decrease in NDVI values along the experimental assay from December 2023 to June 2024. Globally, AMF inoculation had no effect on NDVI for both plant species (Figure 6A and 6B), and no significant differences were detected among treatments before salt treatment ($p > 0.05$). Two months after saline water irrigation, the plants in saline conditions presented NDVI values relatively higher than plants irrigated with freshwater for both *L. daveaui* ($F(1) = 22.413$, $p < 0.001$) and *L. algarvense* ($F(1) = 18.699$, $p < 0.001$). However, this effect tended to disappear over time though persisting longer in *L. algarvense* plants. In *L. daveaui* plants the saline water irrigation effect soon disappeared, although in December 2023 and April 2024 differences seem to exist ($p < 0.05$). Nevertheless, for high confidence levels no significant differences were detected on those dates ($p < 0.01$). At the end of the experiment, no significant differences were observed among non-inoculated or inoculated experimental groups exposed or not to saline water.

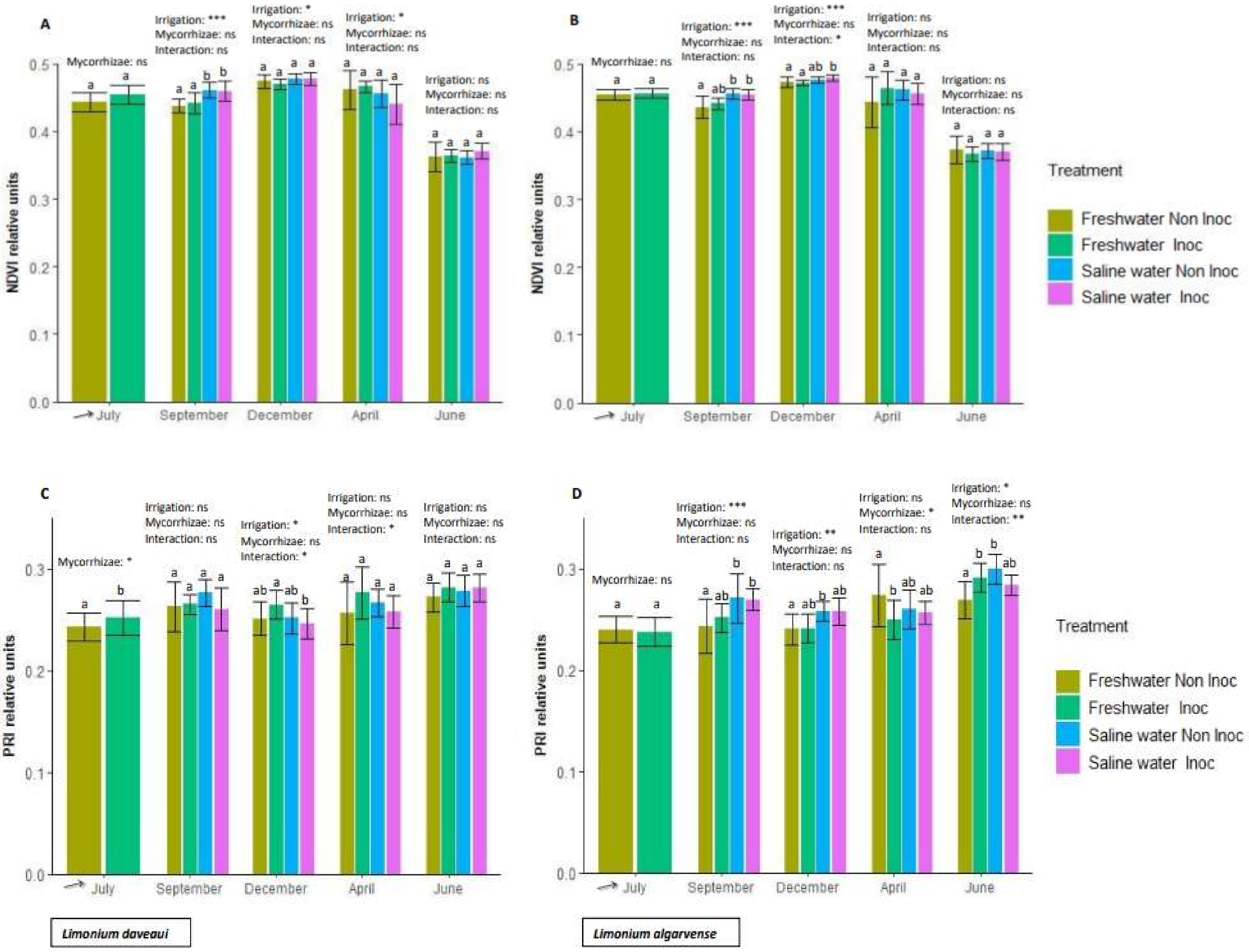


Figure 6 – Physiological parameters measured at different time points in *Limonium daveaui* and in *Limonium algarvense* plants. (**A, B**) Normalized difference vegetation index (NDVI); and (**C, D**) photochemical reflectance index (PRI), measured at different time points in *L. daveaui* (**A, C**) and *L. algarvense* (**B, D**) plants inoculated or not with the autochthonous AMF consortium and irrigated or not with a saline solution (200 mM NaCl). Bars indicate the mean value per treatment ± standard deviation. Different letters at each time point indicate statistical significant differences between means according to the Tukey multiple comparison test. On the top of the bars are the results of the one-way ANOVA (before salt application) and the two-way ANOVA (after salt

application) conducted to study the effect of mycorrhizae inoculation, saline water irrigation and their interaction. A significant effect is indicated through asterisks, “***”, “**” and “*” representing $p < 0.001$, $p < 0.01$ and $p < 0.05$, respectively, and ‘ns’ indicates non-significant differences in a particular measurement time. Arrows indicate the beginning of saline water irrigation. Abbreviations: Non-Inoc – non-inoculated plants; Inoc – AMF inoculated plants.

Contrasting with the results observed for the NDVI parameter, the evolution of PRI from December 2023 to June 2024 showed an increase in the mean values for plants across the different conditions tested. Differences in PRI values were seen among species (Figure 6C and 6D). Before NaCl treatment, *L. daveaui* plants showed significant differences for AMF inoculation ($F(1) = 6.878$, $p < 0.05$) with higher PRI values in inoculated plants than in non-inoculated ones. However, this tendency changed after two months of saline water irrigation, where no effect of AMF inoculation was detected. In December 2023 and April 2024 a significant interaction between irrigation and inoculation treatments was found ($F(1) = 5.000$, $p < 0.05$ and $F(1) = 4.622$, $p < 0.05$, respectively). Inoculated plants had higher mean PRI values in freshwater conditions but lower in saline conditions compared to non-inoculated plants in the same conditions. In June 2024, once again there were no differences between treatments with no effect of AMF inoculation or saline water irrigation.

As for *L. algarvense* there was no effect of AMF inoculation on PRI neither in the beginning of the experiment nor during saline water irrigation. However, there was a significant effect of saline water irrigation two ($F(1) = 17.662$, $p < 0.001$) and five months ($F(1) = 15.477$, $p < 0.01$) after irrigation with saline water, with an increase in PRI values for plants in saline conditions similar to that observed for NDVI values, but once again this effect disappeared over time. In June 2024 there was also a significant interaction between irrigation and inoculation treatment factors ($F(1) = 14.782$, $p < 0.01$). Besides being solely influenced by irrigation treatment factor, higher PRI values were observed for inoculated plants compared with non-inoculated ones in freshwater conditions, differently from what was observed in April 2024. When in saline conditions, AMF-inoculated plants showed the opposite effect with higher PRI values in non-inoculated plants.

Considering stomatal conductance to water vapour-g_s (Figure 7), two months after saline water irrigation no differences were found between inoculated and non-inoculated plants. On the other hand, the irrigation treatment significantly affected this parameter in both *L. daveaui* ($F(1) = 22.572$, $p < 0.001$) and *L. algarvense* ($F(1) = 17.408$, $p < 0.001$). Although in September 2023 plants in saline conditions had higher values than plants in freshwater conditions, in January 2024 g_s values decreased in all plants and the effect of saline water irrigation was found only in *L. daveaui*. In June 2024, a shift in plants responses occurred among the experimental groups and the ones in saline conditions had the lowest values. In *L. daveaui* plants, g_s values differ significantly across irrigation ($F(1) = 57.635$, $p < 0.001$) and inoculation (*F*(1) = 7.182, $p < 0.05$) treatments but no significant interaction between factors was evident. Inoculated plants had higher values than the non-inoculated in freshwater conditions. However, in saline conditions there were no differences associated to AMF inoculation and these plants had the lowest g_s values. Similarly, for *L. algarvense* plants there was a significant effect of both irrigation (*F*(1) = 242.0, $p < 0.001$) and AMF inoculation (*F*(1) = 157.7, $p < 0.001$) treatments as well as their interaction (*F*(1) = 134.0, $p < 0.001$). At this time, a positive effect of AMF inoculation was observed in plants irrigated with freshwater resulting in a significant higher stomatal conductance than in non-inoculated plants. Once again, the lowest g_s values were measured in plants in saline conditions with an interaction indicating a different trend associated to AMF inoculation.

At the end of the experiment, the two-way ANOVA, with species and AMF inoculation as main factors, revealed significant differences for AMF inoculation (*F*(1) = 158.829, $p < 0.001$) in the g_s values of plants under freshwater conditions. Although there was no significant effect of species factor, a significant interaction between factors (*F*(1) = 69.931, $p < 0.001$) was present. For both species in freshwater conditions, inoculated plants had higher values than non-inoculated ones, with *L. algarvense* inoculated plants exhibiting the highest mean g_s values. In saline water conditions, a significant effect was detected among species (*F*(1) = 39.988, $p < 0.001$). Independently of AMF inoculation, *L. daveaui* plants had significantly higher g_s values than *L. algarvense* plants.

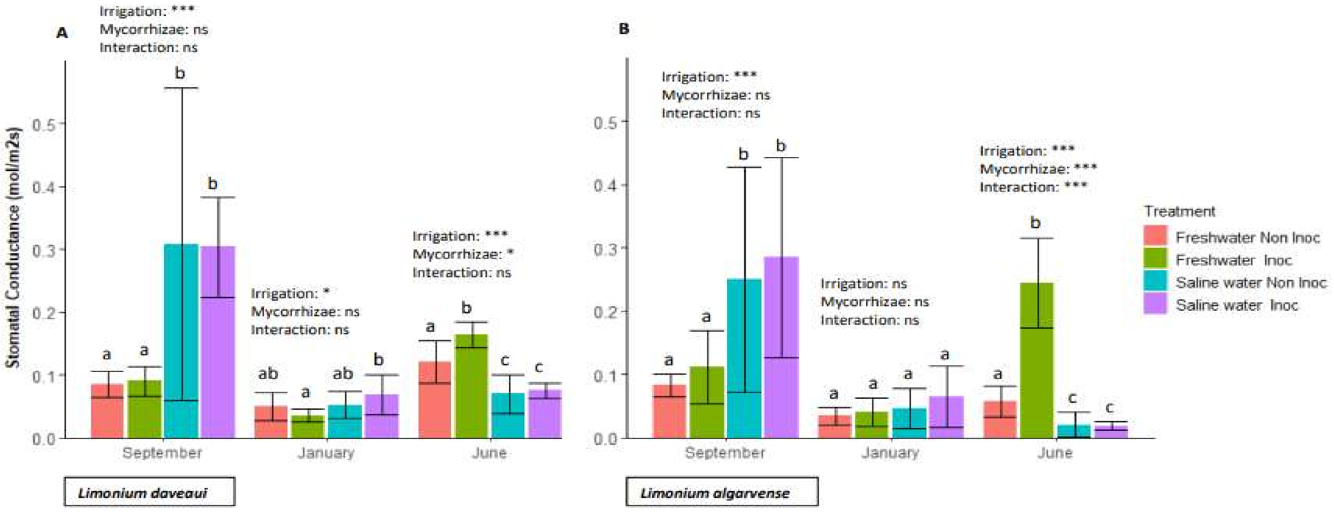


Figure 7 – Stomatal conductance (gs) measured at different time points in *Limonium daveaui* (A) and *Limonium algarvense* plants (B). Plants were inoculated or not with the halotolerant AMF consortium and irrigated or not with a saline solution (200 mM NaCl). Bars indicate the mean value per treatment ± standard deviation. Different letters at each time point indicate statistical significant differences according to the Tukey multiple comparison test. On the top of the bars are indicated the results of the two-way ANOVA conducted to study the effect of mycorrhizae inoculation (AMF), saline water irrigation and their interaction. A significant effect is indicated through asterisks, "***", "**" and "*" representing $p < 0.001$, $p < 0.01$ and $p < 0.05$, respectively, and

‘ns’ indicates non-significant differences in a particular measurement time. Abbreviations: Non-Inoc – non-inoculated plants; Inoc – AMF inoculated plants.

After saline water irrigation, there were visible effects on plant water consumption (Figure 8). Considering all plants maintained in the same greenhouse and under similar conditions, sets of plants with more water in the trays would indirectly represent a smaller quantity of water consumed. There was differential water consumption among plants in different experimental conditions having plants irrigated with freshwater always consumed more water than plants in saline conditions. Besides, there were also differences between inoculated and non-inoculated plants, having the first ones consumed higher quantities when irrigated with freshwater. Even though not so pronounced, this trend was also observed for plants grown in saline conditions. However, in April 2024, a somewhat lower quantity of water was consumed per *L. algarvense* inoculated plants in comparison with non-inoculated plants in saline conditions. The water consumed per plant in June 2024 is not shown since all the water applied was absorbed. Thus, it is not possible to infer about differences between plants in each experimental condition.

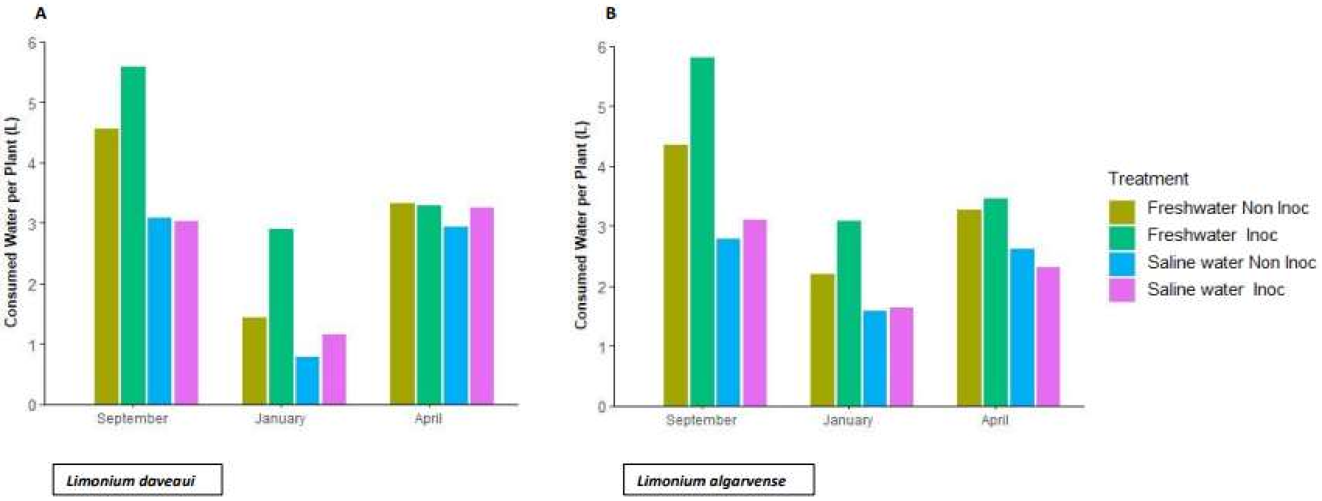


Figure 8 – Mean water consumed monthly per *Limonium* plant. (**A**) *Limonium daveaui* and (**B**) *Limonium algarvense* plants inoculated or not with the consortium of halotolerant AMF and irrigated or not with a saline solution (200 mM NaCl). Abbreviations: Non-Inoc – non-inoculated plants; Inoc – AMF inoculated plants.

Using the collected infrared images (Figure 9), it was possible to analyse plants leaf temperature along the experiment. Before salt treatment, AMF inoculation led to a significant increase in temperature ($p < 0.01$) in both species (Figure 10A and 10B). After two months of irrigation with saline water, *L. daveaui* plants presented a similar pattern with AMF inoculation leading to a significant increase in leaf temperature ($F(1) = 53.496$, $p < 0.001$) for both fresh- and saline water treatments (Figure 10A). The effect of AMF inoculation tended to disappear over time for plants in saline conditions. Even though, the AMF inoculation effect was significant ($p < 0.01$) for plants irrigated with freshwater until the end of the experiment. In April 2024 inoculated plants had lower values than non-inoculated plants with a significant interaction between factors ($F(1) = 9.118$, $p < 0.01$). In *L. algarvense* plants, the AMF effect was significant ($p < 0.05$) for plants in freshwater conditions but not observed for plants in saline conditions (Figure 10B), after two and five months of irrigation with saline water. A significant interaction was also seen in December 2023 ($F(1) = 12.083$, $p < 0.01$) and April 2024 ($F(1) = 35.18$, $p < 0.001$). In April 2024, in freshwater conditions inoculated plants had higher temperature values than non-inoculated plants whereas the opposite pattern was observed for plants in saline conditions. Nevertheless, no significant effect of AMF inoculation was found in April 2024. Even with freshwater irrigation, AMF inoculation effect disappeared at the end of the experiment. Concerning the irrigation treatment, there was a significant effect ($p < 0.001$) during all the experiment and higher temperatures were observed in both species for plants subjected to saline conditions.

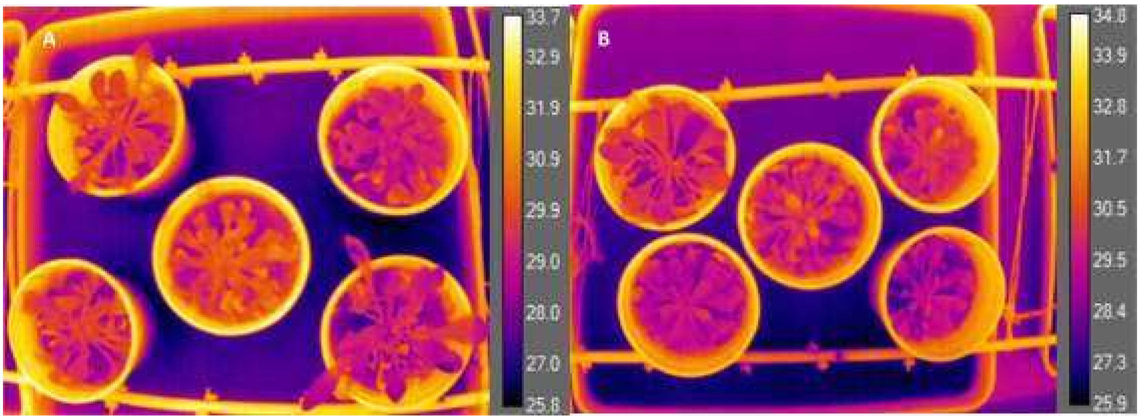


Figure 9 – Infrared images taken two months after saline water irrigation (200 mM NaCl). *Limonium* spp. irrigated with tap (**A**) and saline water (**B**).

Additionally, to understand plant's ability to thermoregulate, the difference between air and leaf temperatures at each measurement time was considered. The room temperature measured in each time point in July 2023, September 2023, December 2023, April 2023 and June 2024 was 41.7 °C, 31.5 °C, 14.2 °C, 19.1 °C and 35 °C, respectively. Figure 10 (C, D) shows that before saline water irrigation plants temperature was significantly above room temperature. This trend changed from September 2023 to April 2024 with the room temperature decrease in these colder months. Once again, in July 2024 room temperature increased and plants maintained lower temperature than the plants' compartment.

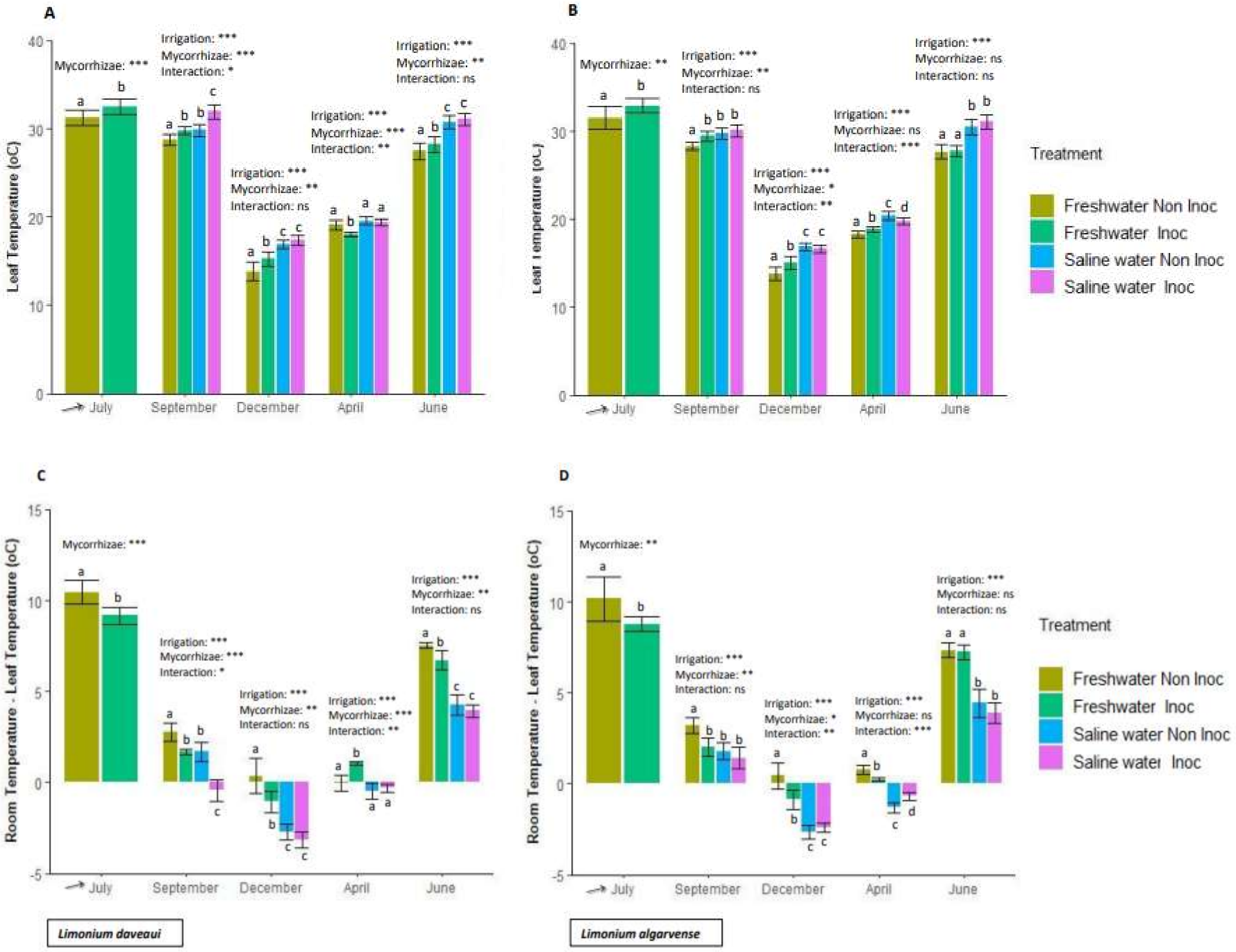


Figure 10 – Leaf temperature measured at different time points and comparison with room temperature. The leaf temperature was obtained from infrared images of *Limonium daveaui* (**A**) and *Limonium algarvense* (**B**) plants inoculated or not with autochthonous AMF and irrigated or not with a saline (200 mM NaCl) solution. Corresponding differences between air and leaf temperature measured at each time point for *L. daveaui* (**C**) and *L. algarvense* (**D**) plants. Bars indicate the mean value per treatment ± standard deviation. Different letters at each time point indicate statistical significant differences according to the Tukey multiple comparison test. On the top of the bars are the results of the one-way ANOVA (before salt application) and the two-way ANOVA (after salt application) conducted to study the effect of mycorrhizae inoculation (AMF), saline water irrigation and their interaction. A significant effect is indicated through asterisks, "***", "**" and "*" representing $p < 0.001$, $p < 0.01$ and $p < 0.05$, respectively, and 'ns' indicates non-significant differences in a particular measurement time. Arrows indicate the beginning of saline water irrigation. Abbreviations: Non-Inoc – non-inoculated plants; Inoc – AMF inoculated plants.

3.2. Effects of AMF Inoculation and Saline Water Irrigation on Plant Vegetative Growth

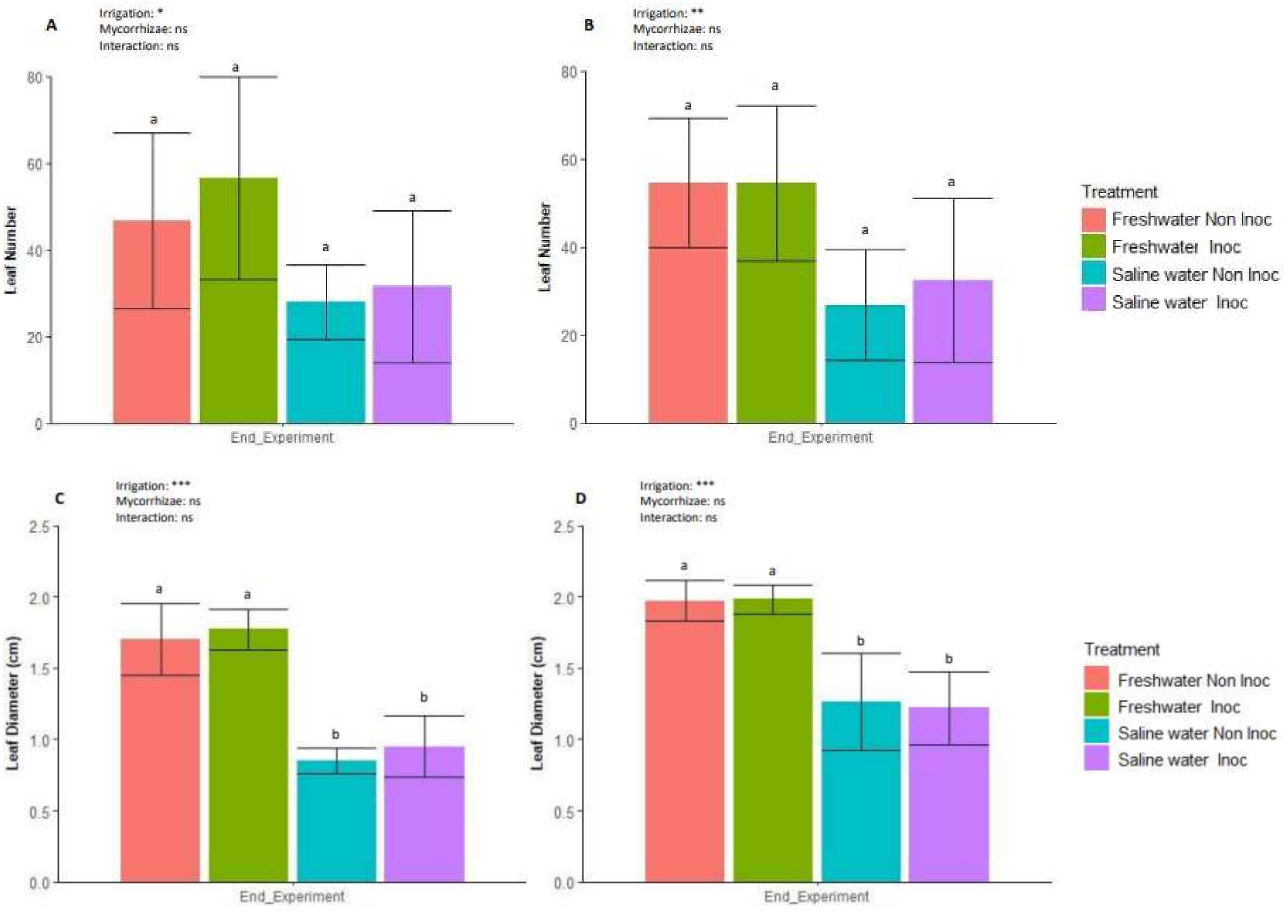
Since significant differences in the measured physiological parameters were already evident in September 2023, this month was selected for comparisons of plant vegetative growth, along with June 2024 (end of the experiment).

The mean number of total fresh and dry leaves for plants in each experimental condition is shown in Table 1. In freshwater conditions, non-inoculated plants had a slightly higher number of leaves than those inoculated with AMF. By contrast, under saline conditions, inoculated plants showed a greater number of leaves than the non-inoculated plants on both dates. By comparing the mean values in September 2023 and June 2024, it was possible to observe an increase in the number of leaves for both species in all treatments. The linear mixed model and the following Tukey test, used to analyse the data collected in those months, revealed a significant effect of Time factor ($p < 0.001$), indicating an increase in the total number of leaves along the experiment.

Table 1 – Mean total number of leaves (fresh and dry), measured in September 2023 and June 2024, along with the corresponding standard deviation. *Limonium daveaui* and *Limonium algarvense* plants subjected to the different experimental conditions, namely inoculation or non-inoculation with AMF, and tap or saline water irrigation. Abbreviations: Non-Inoc – non-inoculated plants; Inoc – AMF inoculated plants.

	Experimental Treatment		**September 2023**	**June 2024**
Limonium daveaui	**Freshwater**	**Non-Inoc**	63 ± 3	121 ± 30
		Inoc	60 ± 4	118 ± 17
	Saline water	**Non-Inoc**	52 ± 9	101 ± 11
		Inoc	73 ± 26	154 ± 72
Limonium algarvense	**Freshwater**	**Non-Inoc**	66 ± 13	104 ± 24
		Inoc	61 ± 21	84 ± 30
	Saline water	**Non-Inoc**	59 ± 15	94 ± 27
		Inoc	61 ± 20	104 ± 37

By the end of the assay, differences were detected in the number of fresh leaves (Figure 11A and 11B) and their diameter (Figure 11C and 11D), as well as in plant diameter (Figure 11E, 11F and 12) among experimental conditions. Although AMF inoculation had no effect on the parameters considered to monitor plant growth, the irrigation water treatment had a significant effect on the number of fresh leaves (although for higher confidence levels and according with the Tukey test results no significant differences were detected), fresh leaves' diameter ($p < 0.001$) and plant diameter ($p < 0.001$) in both species. Plants irrigated with saline water had significantly lower vegetative development than plants cultivated in freshwater conditions, presenting smaller leaf width and shorter leaves. Besides, regarding plant diameter, a significant interaction between irrigation and AMF inoculation ($F(1) = 9.755$, $p < 0.01$) was detected in *L. daveaui* plants. Nevertheless, for higher confidence levels and according with the Tukey test results, no significant differences were found between inoculated and non-inoculated plants.



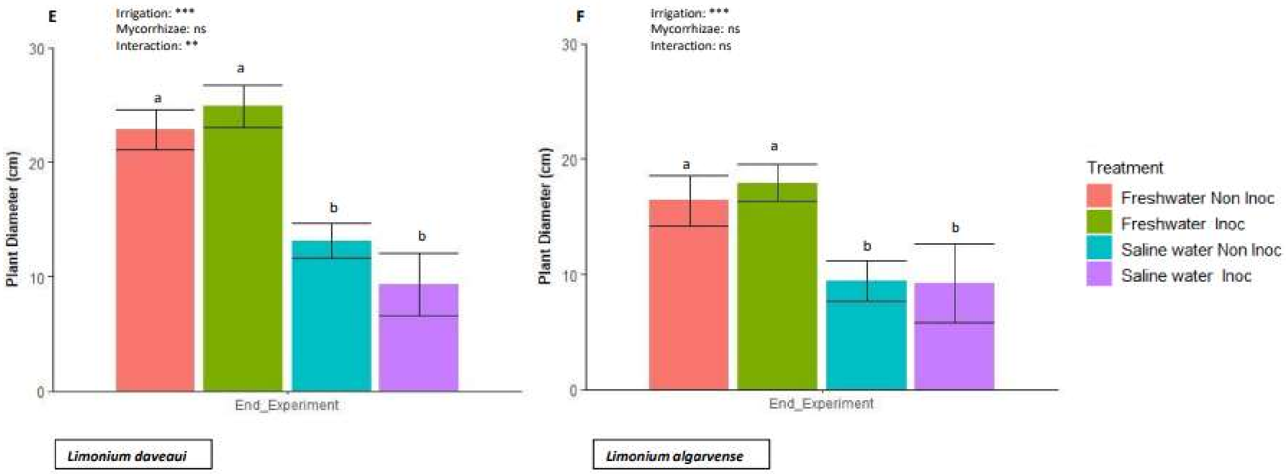


Figure 11 – Plant growth parameters. (**A, B**) Number of fresh leaves; (**C, D**) fresh leaves' diameter; and (**E, F**) plant diameter, considering the fresh leaves, measured at the end of the experiment in *L. daveaui* (**A, C, E**) and *L. algarvense* (**B, D, F**) plants inoculated or not with autochthonous AMF consortium and irrigated or not with a saline solution (200 mM NaCl). Bars indicate the mean value per treatment ± standard deviation. Different letters at each time point indicate statistical significant differences according to the Tukey multiple comparison test. On the top of the bars are indicated the results of the two-way ANOVA conducted to study the effect of mycorrhizae inoculation (AMF), saline water irrigation and their interaction. A significant effect is indicated through asterisks, "***", "**" and "*" representing $p < 0.001$, $p < 0.01$ and $p < 0.05$, respectively, and 'ns' indicates non-significant differences in a particular measurement time. Abbreviations: Non-Inoc – non-inoculated plants; Inoc – AMF inoculated plants.

By the end of the experiment, it was observed that plants grown in saline conditions (Figure 12B and 12D) showed leaf necrosis and a higher proportion of dead leaves than plants developed under freshwater irrigation (Figure 12A and 12C). Moreover, the first plants had a higher level of salt excretion from their leaves (Figure 12B and 12D) than the latter plants. Further, in saline conditions a higher level of salt efflorescence was also found on the soil surface and in the cultivation trays.

Figure 12 – *Limonium daveaui* (A, B) and *Limonium algarvense* (C, D) plants grown under freshwater (A, C) and saline conditions (B, D), one year after cultivation. Plants in saline conditions presented their leaf epidermal surfaces fully covered with salt crystals (indicated by arrows). *Limonium daveaui*'s necrotic leaves are also shown (circled).

3.3. Effects of AMF Inoculation and Saline Water Irrigation on Plant Reproductive Growth

Regarding plants phenology, *L. algarvense* plants developed scapes earlier (March) than *L. daveaui* (April) for plants maintained in both fresh- and saline water conditions. The duration of the reproductive development was longer for *Limonium* plants in saline conditions, with *L. algarvense* and *L. daveaui* plants producing inflorescences in September 2023 and November 2023, respectively. To understand the effect of AMF inoculation and irrigation water in plant reproductive growth, a total of 7940 flowers and 1852 seeds were counted (Table 2). In both species and among all treatments, inoculated plants had the higher % of seeds comparing with non-inoculated plants under the same irrigation regimes. The higher number of plants that developed inflorescences, flowers and seeds was seen in freshwater conditions for *L. daveaui* plants inoculated with AMF and *L. algarvense* non-inoculated plants. However, *L. algarvense* inoculated plants irrigated with freshwater showed higher percentage of seeds. In freshwater conditions, non-inoculated *L. daveaui* plants did not develop inflorescences while inoculated plants had a higher number of flowers and percentage of seeds among all treatments. Still, this effect was not so outstanding in the number of flowers and seeds for *L. algarvense* plants. Irrigation with saline water lowered plants reproductive growth, leading to lower number of flowers and seeds percentage than those of plants grown in freshwater conditions. Even though plants were able to develop inflorescences, the effect was more pronounced in *L. algarvense* with a strong decrease in the analysed parameters when in saline conditions. Remarkably, *L. algarvense* plants developed more inflorescences and had a higher seeds percentage than *L. daveaui*, although this later species produced more flowers.

Table 2 – Flowers and seeds produced in *Limonium daveaui* and *Limonium algarvense* plants subjected to the experimental conditions tested, namely, inoculation or non-inoculation with AMF, and fresh- (tap) or saline water irrigation. The following parameters of plant reproductive growth were considered: total number of plants that produced flowers, total number of dried flowers and seeds, and seed percentage (%). In the calculation of seed %, scapes collected with immature inflorescences were not considered. Abbreviations: Non-Inoc – non-inoculated plants; Inoc – AMF inoculated plants.

	Experimental Treatment		**Number of Flowering Plants**	**Number of dried Flowers**	**Number of Seeds**	**%Seeds**
Limonium daveaui	**Freshwater**	**Non-Inoc**	0	-	-	-
		Inoc	4	2841	593	21
	Saline water	**Non-Inoc**	1	196	7	4
		Inoc	2	1426	202	14
Limonium algarvense	**Freshwater**	**Non-Inoc**	4	1543	680	44
		Inoc	3	898	255	71
	Saline water	**Non-Inoc**	3	505	69	26
		Inoc	3	531	46	33
	Total			7940		1852

4. Discussion

Overall, our results showed a significant effect of saline water irrigation (200 mM NaCl) and inoculation with a halotolerant AMF consortium isolated from the rhizosphere of the endangered *L. daveaui*. Although saline water irrigation have been positive at the beginning of the experiment, continued exposure to saline conditions for one year lead to an impaired plant growth. Regarding microbial inoculation, despite differences in monitoring dates and *Limonium* species physiological responses, the positive effects on plant performance were evident. The enhanced reproductive development in inoculated plants, with higher flower production and seed percentage, was also revealed. Throughout the experiment, negative effects of mycorrhizae inoculation were never detected.

4.1. Inoculation with Autochthonous Arbuscular Mycorrhizal Fungi Improved Plant Performance

In previous studies, *L. algarvense* and *L. daveaui* plants showed reduced values of photosynthetic indexes when grown in salinized substrates and/or irrigated with estuarine water at 100 mM NaCl compared to plants in non-saline ones, indicating an impaired plant physiological status (Cortinhas *et al*., 2020, 2021). Contrastingly, in the present study using saline water at 200 mM NaCl, plants initially had higher NDVI values for both species and higher PRI values for *L. algarvense* (Figure 6), representing a positive effect of saline water irrigation. This effect tended to disappear over time, and the observed values were similar between fresh- and saline water cultivated plants. As expected for halophyte *Limonium* spp., plants tolerated high concentration of NaCl (200 mM) as described for other halophytes species under saline conditions (Flowers and Colmer, 2008, 2015). The current study moreover shows that plants were able to grow under repeated exposure to saline water irrigation for one year. Comparing with glycophyte plants such as grapevines, an exposition to saline solution at 75 mM during six days caused salt stress and decreased plant growth and survival (Navarro-Torre *et al*., 2023b).

Overall, AMF inoculation had no effect on NDVI and PRI values for both plant species. Another study reported higher NDVI and PRI values in *L. algarvense* plants inoculated with non-native AMF (*Rhizoglomus irregulare* BEG72) after three months in saline conditions (Nogales *et al*., 2023). When *L. algarvense* plants were double-inoculated with a consortium of plant growth-promoting bacteria and non-native AMF, positive results were seen on plants performance in saline conditions at 100 mM NaCl, with higher PRI and NDVI values than those observed in plants inoculated only with AMF or when in non-saline substrates (Nogales *et al.*, 2023). The use of microbial technologies has been widely described in several crops (e.g., soybean, pepper, maize, olive trees) and non-crop (e.g., *Asteriscus maritimus*) species as a successful tool to alleviate plant stress and increase productivity in salt-affected soils (Navarro-Torre *et al.*, 2023a). Among halotolerant microorganisms, those belonging to the subphylum Glomeromycotina are the most commonly occurring mycorrhizal fungi in salty environments to which they are adapted, enabling them to facilitate plant growth and protecting from salinity negative impacts (Zahir *et al.*, 2019). Positive effects of AMF inoculation were mainly seen in freshwater conditions but not in saline conditions after one year exposure to saline water irrigation. It is worth noting that the characteristics of the tap water used in this study were not analysed. Thus, we suggest an AMF differential behaviour depending on the intensity of the salt treatment. In freshwater conditions, AMF inoculation with *L. daveaui* autochthonous strains had a positive effect in *L. daveaui* PRI values since the beginning of the experiment. As for *L. algarvense* plants grown in freshwater conditions, the positive effects of AMF inoculation on PRI values were only detected by the end of the experiment. Differences between *Limonium* species physiological responses after inoculation are not unexpected considering that the halotolerant AMF were isolated from autochthonous *L. daveaui* plants. Although no convincing evidence has been presented demonstrating that AMF are host-specific, host preferences and host selectivity have been widely reported (van der Heijden *et al.*, 2015), with differences in fungal behaviour or characteristics in interacting with hosts (Tian *et al.*, 2004). Thus, it would be plausible to consider a preference of AMF to *L. daveaui* plants and a faster establishment of the symbiotic relationship even though conferring benefits for both species over time.

When combined, NDVI and PRI can provide information regarding photosynthetic phenology and activity, with the exact use depending upon the application and time frame (Gamon *et al.*, 2015). In the current study, differences were detected in both parameters, with a decrease in NDVI values from December 2023 to June 2024. Similar results were previously observed for *L. daveaui*, where NDVI values dropped significantly from late spring to autumn for plants in saline conditions (Cortinhas *et al.*, 2021). The NDVI is an indicator of chlorophyll content, phosphorus and nitrogen nutrition, commonly used to predict biomass and plant vigor (Sembiring *et al.*, 1998; Garbulsky *et al.*, 2011). The decrease in this parameter values could be associated to leaf senescence that normally occurs when plants start flowering, but also with plant nutrient status (Sembiring *et al.*, 1998; Cortinhas *et al.*, 2021). A decrease in plant nutrient status may have occurred as a result of nutrient consumption along the experimental assay, particularly during flowering season (Figure 5), and their utilization by microorganisms to support growth and metabolic activities (Nogales *et al.*, 2023). Additionally, differences in carbon gain might be determined by differences in self-shading

(Conesa *et al.*, 2019) that could be increased along the experiment with plant growth and increasing number of leaves. Contrastingly, the evolution of PRI from December 2023 to June 2024 showed an opposite trend with an increase in the mean values for plants in all experimental conditions. This could be related to seasonal variation, since in summer months higher temperature and longer photoperiod can influence photosynthetic light use efficiency. An increase in PRI can be coincident with a period of spring transition associated with photosynthetic activation and increasing chlorophyll: carotenoid ratios (Gamon *et al.*, 2015).

Stomata allow the exchange of gases, most importantly water vapour and CO_2 between the interior of the leaf and the atmosphere by controlling the aperture of the stomatal pore and the number of stomata that form on the epidermis (Hetherington and Woodward, 2023; Buckley, 2017). Stomatal control is an effective response in protecting plants against the impacts of water content decrease and together with leaf growth inhibition are the earliest responses in water-deficit conditions (Cochard *et al.*, 2002; Chaves *et al.*, 2003; Hugalde and Vila, 2014; Acosta-Motos, 2017; Morton *et al.*, 2019). In our study, plants were constantly supplied with water and drought conditions were absent. However, the presence of water-soluble salts reduces the water potential of the soil, bringing it closer to the water potential in the plant roots, causing drought stress for the plants (Ayers and Westcot, 1985; Stavi *et al.*, 2021).

Initially, as shown in Figure 7, saline water irrigation had positive effects on g_s leading to higher values than in plants maintained in freshwater conditions, as found for NDVI and PRI parameters. Nevertheless, by observing the mean values for water consumed per plant in the same month (Figure 8), it was not possible to see a direct correlation with g_s values since plants in freshwater conditions seem to consume more water than those in saline conditions. Therefore, it seems more reliable only to compare plants in the same irrigation treatments. Increasing water salinity reduces evaporation since the dissolved salt ions lower the free energy of the water molecules (Salhotra *et al.*, 1985; Biazar *et al.*, 2020). If saline water evaporation was less than that from the freshwater irrigation, and taking into account the air temperature registered inside the greenhouse in September 2023 (~ 31.5 ^{o}C), there would be a higher quantity of non-absorbed saline water and lower quantity of non-absorbed freshwater. Consequently, the mean values of freshwater consumed would be higher than that of saline water. Thus, to compare the quantity of water consumed between irrigation treatments the rates of evaporation should be estimated considering both types of irrigation water.

Indeed, the mechanisms that control opening and closing and development of stomata are influenced by a complex set of factors, ranging from light intensity to atmospheric CO_2 concentration in addition to internal factors such as leaf water status and endogenous plant hormones (Cochard *et al*., 2002; Hetherington and Woodward, 2023; Chaves *et al*., 2003; Buckley, 2017). The lower values detected in the beginning of 2024 among plants cultivated in the different conditions are probably due to environmental factors recorded in winter months, with low light intensity, high air humidity (around 66.8% in January 2024) and low evaporative demand, associated to the low g_s values recorded. Contrastingly, by the end of the assay irrigation with saline water had a negative effect on plants g_s values, which significantly decreased. This finding is probably associated with an impaired physiological status, cumulative saline water irrigation effects and evident salt efflorescence in soil surfaces and in cultivation trays. Since stomata regulate photosynthesis (Hetherington and Woodward, 2023; Buckley, 2017), the drawback of the stomatal closure for plants is the lowered carbon gain and the impaired growth and reproductive success (Cochard *et al*., 2002; Chaves *et al*., 2003). Remarkably, besides the low g_s values found in June 2024 in plants irrigated with saline water, the PRI and NDVI values were similar among plants cultivated in the different irrigation regimes. In *Limonium* spp. a higher Rubisco specificity factor (relative capacity to catalyze carboxylation and oxygenation of ribulose 1,5-bisphosphate) was described when compared with other C3 plants (Galmés *et al*., 2005, 2007). Thus, *Limonium* plants could possibly sustain a somewhat higher photosynthesis with stomata almost totally closed (Galmés *et al*., 2005, 2007).

Isohydric plants deal with stress conditions by closing stomata as necessary to limit transpiration, allowing the plant to keep leaf water potential at constant ranges and maintaining high plant relative water content (Sade *et al*., 2012). Stomata closure response could be an indicative of salt stress in *Limonium* plants maintained in saline conditions, resembling an “isohydric” pattern. To the best of our knowledge this is the first time that an isohydric response is described for *L. daveaui* and *L. daveaui* species. The same behaviour has previously been reported for other *Limonium* spp. when in drought conditions (Galmés *et al*., 2007). *Limonium* species may be considered as drought-avoidant maintaining leaf water potential and relative water content at relative high values (Galmés *et al*., 2007). In saline conditions *L. algarvense* plants had lower g_s values than *L. daveaui* plants. These findings could be related to the different positions occupied by both species in saltmarshes. Both *Limonium* species thrive in priority habitat 1510* Mediterranean salt steppes (*Limonietalia*) (European C., 2013), inhabiting soils periodically flooded by saline water and experiencing extreme summer drying, often characterized by salt efflorescence (European C., 2013). Nonetheless, *L. algarvense* thrives in the intertidal zone of saltmarshes while *L. daveaui* is only inundated in equinoctial tides at the margins of marsh slopes, on the banks of embankments and saltmarsh walls of *Salinas* (Costa *et al*., 2014; Caperta and Carapeto 2020; Cortinhas *et al*., 2020, 2021). Considering these assumptions, *L. daveaui* might be able to tolerate higher salinity levels and thus maintaining higher g_s values. Physiological differences inherent to species should be considered, namely, possible differences in the number of salt glands (Caperta *et al.*, 2020) and stomata that might affect stomatal conductance values and influence salt tolerance.

Regarding the effect of AMF inoculation on g_s values, even if in general no significant differences were detected throughout the assay, plants inoculated with AMF tended to have higher g_s mean values than non-inoculated plants (Figure 7). For *L. daveaui* plants in saline conditions, in January 2024 it was observed a higher stomatal conductance in AMF inoculated plants. A smaller reduction in g_s values under salinity conditions has been described in AMF plants from glycophyte species (e.g., maize, rice) and salt-tolerant species (*Robinia pseudoacacia*) (Zahir *et al.*, 2019; Sharma *et al.*, 2021). The positive effect of AMF inoculation indicates a better plant water status, enabled by "maintained" CO_2 diffusion through stomata (Sharma *et al.*, 2021). By the end of the experiment, AMF inoculation had positive effects for both species leading to significant higher g_s values for plants grown in freshwater conditions. Therefore, our results suggest that AMF inoculation seem to favour stomata aperture probably as a result of a better plant water status and water use efficiency, by aiding water uptake (Porcel *et al.*, 2012; Kapoor *et al.*, 2013; Zahir *et al.*, 2019). The induced root proliferation and the sturdier vascular system in AMF plants are associated to higher transpiration rates and stomatal conductance (Kapoor *et al.*, 2013; Zhu *et al.*, 2022). These results are in accordance with the mean values of water consumed monthly per plant (Figure 8) evidencing that AMF increases water consumption in inoculated plants, especially when in freshwater conditions. Several studies demonstrate that AMF can promote plant water absorption and water content in horticultural crops via passive and active water transport in plant roots (Zahir *et al.*, 2019; Zhu *et al.*, 2022). In saline conditions AMF inoculation also seems to increase water consumption but our observations were not so consistent, as in April 2024 inoculated *L. algarvense* plants irrigated with saline water consumed less water than non-inoculated plants. Despite the mean values of water consumption for June 2024 not being available for comparison, by the end of experiment AMF inoculation had no significant effect in g_s values for both species when in saline conditions. Environmental factors such as temperature, resource availability, environmental stresses and the biotic composition of the surrounding community or host ontogeny can all affect the distribution of costs and benefits incurred by the host and microbial symbionts (Leung and Poulin, 2008; Drew *et al.*, 2021; El-Metwally *et al.*, 2023). At highly stressful conditions, due to the reduced plant C assimilation and the high C costs that endophytic microorganisms impose to their hosts, mutualistic symbioses may also turn into commensalistic or even parasitic in response to even the slightest environmental change

(Leung and Poulin, 2008; Estrada *et al*., 2013; van der Heijden *et al*., 2015; Navarro-Torre *et al*., 2023b). Even if in the present study no negative effects were detected in AMF inoculated plants, the duration of saline water irrigation might have caused negative impacts on the symbiotic relationship.

Leaf temperature can be used to predict several plant environmental constraints and monitor plant physiology for early stress detection (e.g., drought and salt stress) (Costa *et al*., 2010; Esmaeili *et al*., 2016; Navarro-Torre *et al*., 2023b) since it is correlated with several processes (e.g., stomatal conductance, leaf transpiration, photosynthesis rates) and influenced by environmental factors like air temperature (Jones *et al*., 2002, 2004; Greer, 2012). For example, in saline conditions salt-tolerant wheat cultivars with higher mean shoot dry matter maintained lower increase in the mean leaf temperature whereas salt-sensitive cultivars had lower shoot growth and higher leaf temperature (Esmaeili *et al*., 2016). Salt stressed grapevine plants also had higher temperature values than control plants in freshwater conditions (Navarro-Torre *et al*., 2023b). Hence, high leaf temperature values could be expected in *Limonium* salt-stressed plants. Our study includes the first tentative to use thermal images to analyse plant performance and predict plant stress in *Limonium* plants under different irrigation regimes. Before irrigation with saline water, AMF inoculated plants presented higher leaf temperatures than non-inoculated ones (Figure 10A and 10B). Since leaf temperature can be correlated with photosynthesis, we assume this increase in temperature values as a positive effect of AMF inoculation. Also, from autumn 2023 to spring 2024 (September, December 2023 and April 2024), inoculated plants grown in freshwater conditions presented smaller (even negative) air and leaf temperature differences than those measured in non-inoculated ones (Figure 10C and 10D). These findings revealed that plants can maintain high leaf temperatures despite the low air temperatures during the colder months. Thus, the inoculation with AMF benefited plants' ability to thermoregulate. Also, plants subjected to saline water irrigation had higher leaf temperatures than plants in freshwater conditions (Figure 10). Considering temperature results together with those for NDVI, PRI and stomatal conductance parameters (Figure 6 and 7), plants in saline conditions had a better performance from autumn 2023 to spring 2024. Two months after saline water irrigation a similar trend was seen in AMF-inoculated *L. daveaui* plants, reinforcing another positive effect of AMF in saline conditions. Nevertheless, in saline conditions AMF inoculation did not affected *L. algarvense* plants' temperature and it disappeared over time in *L. daveaui* plants.

Despite the apparently beneficial effects of applying 200 mM NaCl, by the end of the experiment (summer 2024), the high leaf temperature values observed for plants in saline conditions, as well as the low stomatal conductance (Figure 7) measured at the same time

(June 2024), strongly represent a salt stress pattern. In estuarine grasses, decreasing water potential (due to drained soil and high salt concentrations) led to reduced transpiration rates and, consequently, a decrease in latent cooling, resulting in an increase of up to 4°C in leaf temperatures (Maricle *et al*., 2007). Stomata aperture plays a role in leaf cooling through latent heat exchange, thus, its closure can lead to an increase in plants leaf temperature (Chaves *et al*., 2003; Acosta-Motos, *et al*., 2017; Navarro-Torre *et al*., 2023b). Although, leaf temperature *per se* does not allow us to conclude the level of stress in *Limonium* plants it rather supports other physiological results.

4.2. Long-term Exposure to Saline Water Negatively Affected Plant Vegetative Growth

Concerning plant growth parameters, the mean values of total leaf number tended to be higher for both species in AMF inoculated plants irrigated with saline water (Table 1). However, within the same treatment highly variable values were seen among plants, thus, our results did not allow us to conclude about AMF inoculation benefits. Other effects eventually inherent to each individual may be masking probable mycorrhizae effects. In any case, after one year of saline water irrigation, AMF inoculation had no effect on final plant growth. Similarly, in other studies *Limonium* AMF inoculated plants did not show any significant effect in growth parameters such as number of leaves and shoot fresh biomass (Nogales *et al*., 2023). The beneficial effect of AMF may be seen in improving plant tolerance to salinity and survival in natural environments, but not necessarily in an enhanced plant growth, which potentially depends upon other constitutive factors (Montserrat-Martí et al., 2011; Camprubi et al., 2012).

Differences were only detected between plants grown under different irrigation conditions. Previously it was demonstrated that *L. daveaui* plants had lower development when grown in salinized substrate at 100 mM NaCl (Cortinhas *et al*., 2021). A similar trend was observed for *L. algarvense*, where irrigation with saline water (100 mM NaCl) strongly affected growth leading to plants with fewer and smaller leaves compared to those freshwater irrigated and with a better growth performance (Cortinhas *et al*., 2020; Rodrigues *et al*., 2020). In our study, and according to the parameters studied, plant growth was affected by saline water irrigation, having those plants shorter green leaves with smaller leaf width, leaf necrosis and higher proportion of death leaves than freshwater irrigated ones (Figure 11 and 12). The decrease in leaf area through reduced growth and shedding of older leaves is one of the traits involved in minimising water loss and allowing the reallocation of nutrients stored in older leaves to the stem or younger leaves (Chaves *et al*., 2003). The effect of salinity on growth varies amongst halophytes being able to grow in saline conditions while others also grow with low salinity or in the absence of salt (Flowers and Colmer, 2008; Grigore *et al.*, 2012; Rodrigues *et al.*, 2020). Halophytes are also subjected to the wide range of possible deleterious effects of salt, such as interference with nutrients and water absorption, reduced carbon fixation, alteration of osmotic balance, inhibition of enzymatic activities and essential cellular processes, oxidative stress (Ayers and Westcot, 1985; Flowers and Colmer, 2008; Morton *et al.*, 2019). Together with the high ions' toxicity, plant growth inhibition and decline can be explained for sufficiently high salinity levels (Flowers and Colmer, 2008; Grigore *et al.*, 2012). Nonetheless, halophytes developed several strategies to cope with salinity, among them the presence of salt glands to excrete the excess of salt as typically found in *Limonium* species (Caperta *et al.*, 2020) and as observed in this study (Figure 12). This adaptation contributes to *L. daveaui* and *L. algarvense* salt tolerance at high NaCl concentrations and during an extended period (one year).

4.3. Microbial Technologies Improved *Limonium* Reproductive Growth

Besides affecting vegetative growth, irrigation with saline water also seems to affect reproductive growth. Regarding plants' phenology, plants subjected to saline conditions did not present the predicted timing for reproductive development illustrated in Figure 5. In the beginning of September 2023 and by the end of November 2023 the inflorescences were still present in *L. algarvense* and *L. daveaui* plants, respectively. In salinized substrates *L. daveaui* flowering stems emerged later than those grown in non-saline substrates (Cortinhas *et al.*, 2021). In our study, salinity seemed to extend the flowering duration in both *Limonium* species plants reproduction without a later emergence of flowering stems. In other halophytes species, changes in flowering time under appropriate salinity have been reported, for instances, longer flowering duration under saline as compared to non-saline conditions (Yuan *et al.*, 2019). Furthermore, for both species, a higher number of scapes and more flowers were reported in plants grown in non-saline conditions compared to those subjected to saline water irrigation (Cortinhas *et al.*, 2020, 2021; Rodrigues *et al.*, 2020). In this study, saline water irrigation lowered plant reproductive growth, leading to lower number of flowers and seeds and lower seed percentage (Table 2), being this effect more pronounced in *L. algarvense*.

The few studies examining the effects of AMF inoculation on plant reproduction for some horticultural crops (e.g., *Chrysanthemum morifolium*, *Gazania rigens*, tomato) report an increase in flower size and number, flowering time, pollen and nectar numbers, pollen germination and pollen tube growth (Zhu *et al.*, 2022). In *L. algarvense*, halotolerant AMF inoculation also increased the fresh biomass of inflorescences in plants grown in saline soils irrigated with brackish water (Nogales *et al*., 2023). In our study, AMF inoculation favoured plant reproductive growth leading to an increase of up to 27% in the seed percentage compared with non-inoculated plants in the same conditions (Table 2). This trend was observed among all treatments and for both species. Hence, these findings highlight that AMF inoculation improves plants reproduction and successful seed formation. It was previously found that *L. algarvense* produces heterogeneous pollen in morphology and size, with moderate-to-no viability that poorly germinates *in vitro*, but with a high percentage of seeds indicating a probable asexual origin through apomixis (asexual seed production) (Conceição *et al*., 2019). The self-incompatibility system linked to pollen-stigma dimorphisms preventing selfing (Róis *et al*., 2016) was confirmed for the studied species which are self-incompatible (Gomes-Domingues *et al*., in preparation). Given that in the present study the inflorescences were covered with caps since the beginning of flowering season to prevent possible crosses between individuals, and considering that both species are self-incompatible, our findings support that they produce clonal seeds. Additionally, differences between species were detected, with *L. algarvense* plants developing more inflorescences with a higher percentage of seeds (25.65 – 71.03%) than *L. daveaui* plants (3.57 – 20.87%). Moreover, this study supports that microbial technologies using halotolerant AMF consortium could be an eco-friendly approach to improve *Limonium* spp. reproduction in saline conditions.

5. Conclusions and Perspectives

In conclusion, halotolerant AMF native from a saline environment, to which they are adapted, can be used for *Limonium* species inoculation resulting in beneficial aids for plants cultivated under greenhouse conditions with both tap and saline water irrigation.

This study revealed that irrigation with saline water (200 mM NaCl) improved plant performance until some extent, leading to higher NDVI, PRI and g_s values in the beginning of the experiment. However, after one year in saline conditions, plants showed an isohydric response with lower g_s values and higher leaf temperatures than those measured in freshwater irrigated plants. The long-term exposure to high salinity conditions, evidenced by the presence of salt efflorescence on the soil surface and in the cultivation trays, led to a decreased plant vegetative and reproductive growth. Even though, recretohalophyte *Limonium* spp. can be cultivated, surviving and completing their life cycle, in highly saline conditions during at least one year. The presence of salt crystals covering leaf epidermal surfaces, resulting from salt excretion through salt glands, was observed in plants under saline conditions.

Regarding the AMF consortium, in general, inoculated plants showed higher NDVI, PRI and g_s values, along with higher leaf temperatures even when the room temperature was low, suggesting improved thermoregulation ability, and consumed more water than non-inoculated plants in both experimental conditions. Nevertheless, these effects tended to disappear by the end of the experiment when in saline conditions at 200 mM NaCl. The present study supports that AMF inoculation increases flower and seed production, in both irrigation conditions, without interfering with the plant's ability of asexual seed formation (apomixis). Thus, this microbial technology could be used to cultivate *Limonium* spp. with economic interest, namely for ornamental and pharmaceutical purposes. In a context of water scarcity, the use of saline water is a suitable option in greenhouse conditions and successfully applied in these species cultivation. Together with plants inoculation with a halotolerant AMF consortium isolated from a saline reference ecosystem (e.g., a saltmarsh), a sustainable strategy is described for *Limonium* cultivation, improving plant performance and reproductive growth.

Further studies are important to confirm the benefits of the inoculation with the halotolerant AMF consortium in various species and to prove it as a successful tool to protect and alleviate plants from the negative impacts of salinity. Therefore, in the future, the inoculation with the halotolerant AMF consortium used in this study should be applied, for example, to *Limonium* spp. varieties cultivated for ornamental purposes, as well as to glycophyte species.

Since both *Limonium* species studied are afforded conservation status, the use of native halotolerant AMF could be rewarding in future *in situ* reintroduction studies, namely due to an increase production of seeds potentially contributing to the successful establishment of self-sustaining populations. This experiment established principles for the subsequent reintroduction of *Limonium* species into their natural habitats, using salinity pre-treatments (priming) and transplanting the studied plants into the recipe habitats. Simultaneously, contributing to the conservation of valuable coastal ecosystems, such as saltmarshes, currently in decline and subjected to modifications due to human activities and aggravated by climate change. Finally, being some *Limonium* species CWR representing genetic resources that could provide understanding about the molecular regulation of processes with agricultural applications (e.g., salt tolerance, Sharma *et al*., 2021; apomixis, Caperta *et al*., 2023), further studies should be conducted on candidate genes that could be transferred and genetically engineered in other crop species.

6. References

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