Endophytic mycobiota of *Festuca rubra* subsp. *pruinosa* and its functionality

ERIC CARVALHO PEREIRA

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Endophytic mycobiota of
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Eric Carvalho Pereira

Directed by:

Dr. Iñigo Zabalgogeazcoa
Dra. Beatriz R. Vázquez de Aldana
Dr. Juan B. Arellano Martínez

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Dr. Iñigo Zabalgogeazcoa, Dra. Beatriz R. Vázquez de Aldana y Dr. Juan B. Arellano Martínez, investigadores del Grupo de Investigación de Interacción Planta-Microorganismo, en el Instituto de Recursos Naturales y Agrobiología de Salamanca (IRNASA) del Consejo Superior de Investigaciones Científicas (CSIC).

CERTIFICAMOS:

Que la presente Memoria titulada "**Endophytic mycobiota of *Festuca rubra* subsp. *pruinosa* and its functionality**", ha sido realizada en el Grupo de Investigación de Interacción Planta-Microorganismo, en el Instituto de Recursos Naturales y Agrobiología de Salamanca (IRNASA) del Consejo Superior de Investigaciones Científicas (CSIC), bajo nuestra dirección, por **D. Eric Carvalho Pereira**, y cumple las condiciones exigidas para optar el grado de Doctor con Mención Internacional por la Universidad de Salamanca.

Para que así conste, firmamos el presente certificado en Salamanca a 29 de junio de 2021.

Fdo: Dr. D. Iñigo Zabalgogeazcoa   Fdo: Dra. Dña. Beatriz R. Vázquez de Aldana

Fdo: Dr. D. Juan B. Arellano Martínez

Aos meus queridos pais...

"The highest activity a human being can attain is learning for understanding, because to understand is to be free. He alone is free who lives with free consent under the entire guidance of reason"

- Baruch Spinoza

ACKNOWLEDGEMENTS

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Chapter 1

General introduction

"Microbes add their properties to those of plants to allow them to function normally: above all we have envisioned a world of symbiosis "à l'auberge espagnole (at the Spanish inn)" where each one brings pre-existing capacities ..."
Marc-Andrè Selosse, Jamais Seul (2017)

"Without this fungal web my tree would not exist. Without similar fungal webs no plant would exist anywhere. All life on land, including my own, depended on these networks."
Merlin Sheldrake, Entangled Life (2020)

1.1. Plants are not alone

As Marc-Andrè Selosse and Merlin Sheldrake state, plants are a nest of microorganisms, a whole hidden world full of exciting possibilities and options, with great importance not only for the plant but for an entire ecosystem, for the entire World!

In nature, plants host a diverse community of microorganisms including bacteria, fungi, protozoa, archaea, and viruses, known as microbiome (Trivedi et al., 2020). These microbes can live either inside (endosphere) or outside (episphere) of plant tissues (Turner et al., 2013). Interactions between plants and their associated microbial communities are not unidirectional and can exhibit a wide range of interactions, covering the full range of ecological possibilities, that can either have beneficial (mutualism), neutral (commensalism), or deleterious (pathogen) impact on plant fitness (Hassani et al., 2018). Thus, microbiota can influence the physiology, growth, and health of the host plant to such an extent that plants can form with their associated microbiota single holobionts, whereby evolutionary selection between plants and microorganisms contribute positively or negatively to the overall stability of the system (Vandenkoornhuyse et al., 2015).

Virtually all tissues of a plant harbor highly complex microbial assemblages, mainly bacteria and fungi, that are involved in major plant functions *i.e.*, promoting the productivity and health of the plants in natural environments (Hassani et al., 2018). The establishment and structure of microbial communities on plants are not random but rather controlled by specific assembly rules and several factors (Trivedi et al., 2020). The presence of a subset of microbial lineages designated as core microbiome is strongly associated with a certain host plant species,

independently of soil and environmental conditions (Toju et al., 2018). Thus, the core microbiome involves fundamental microbial taxa that are important for plant fitness and also play an essential role in organizing the assembly of plant-associated microbiomes within and around host plants containing essential functions genes for the fitness of the plant holobiont (Lemanceau et al., 2017; Toju et al., 2018).

1.2. An old alliance between plants and fungi

Fungi are an important component of the plant microbiome. These microorganisms are highly diversified at a structural and functional level, and adopt different trophic strategies to colonize plants. Fungi can colonize both above and belowground plant tissues, mainly belonging to two major phyla: Ascomycota and Basidiomycota (Naranjo-Ortiz and Gabaldón, 2019). The origin of fungi is much older than that of plants; while vascular plants are believed to have originated 400-500 million years ago (Morris et al., 2018), fungi may have originated over 1000 million years ago (Loron et al., 2019). The interaction between plants and fungi is believed to date back to 400-500 million years ago (Lutzoni et al., 2018; Strullu-Derrien et al., 2018). This coincides with the period in which vascular plants evolved, so colonization of the land by plants may have occurred with the help of fungal association (Lutzoni et al., 2018; Strullu-Derrien et al., 2018).

In a natural environment, plants form a close and beneficial association with many fungi that affect their growth, performance and survival. There exists a wide variety of symbiotic plant-fungal interactions. The arbuscular and ectomycorrhizal fungi have been mostly studied, but recent community profiling data indicate that endophytic fungi also represent an important fraction of the fungal microbiota. The symbiotic association with fungi has been reported to offer various benefits to their host plant, such as enhanced mineral nutrients and water uptake, increased tolerance in stressful environments and protection against pathogens (Bonfante and Genre et al., 2010; Thambugala et al., 2020; Decunta et al., 2021).

1.3. Fungal endophytes: a hidden secret within plants

The term endophyte derives from the Greek *endon* = within, and *phyton* = plant. The first description of endophytes dates from the 19th century (Link, 1809). Link described endophytes as a distinct group of partly parasitic fungi living in plants, followed by Bary (1866) who used the term to define any organism that grows within plant tissues. Since then, the term

endophyte has been widely used for any organism found in living plant tissues, including foliar pathogens and mycorrhizal root symbionts, and posterior definitions have been debated by several researchers (Hardoim et al., 2015). Currently, the term endophyte has appeared frequently to describe microorganisms that spend some part of their life cycle, entirely within plant tissues, but do not cause harmful symptoms in the plant host (Wilson, 1995; Hardoim et al., 2015).

Different groups of microorganisms are associated with plant endophytes, including fungi, bacteria, actinomycetes and mycoplasmas (Gouda et al., 2016). Additionally, endophytes can be divided into two different subgroups, such as obligate and facultative. Those endophytes that depend on the metabolism of plants for survival, being spread between plants by vertical transmission, are designated obligate endophytes. Facultative endophytes are those that live freely in soil and colonize plant tissues during some stage of their life cycle (Hardoim et al., 2015). The symbiotic association with endophytes benefits plants, playing an important role in plant disease resistance, synthesis of secondary metabolites, plant growth promotion and increase tolerance to stressful conditions (Hardoim et al., 2015).

Fungal endophytes are a particularly important group of fungi that live a multifaceted lifestyle ranging from mutualists, which improve host plant fitness, to latent pathogens, which do not express any disease symptoms, or saprophytes, which are inactive until tissue or host senescence (Rodriguez et al., 2009; Sánchez Márquez et al., 2012). Fungal endophytes are ubiquitous, and colonize inter and intracellular spaces cell of the host plant (Figure 1.1). They have been isolated from all major terrestrial plant lineages, and in contrasting environments, such as hot deserts, Arctic tundra, mangroves, temperate and tropical forests or grasslands (Arnold and Lutzoni, 2007; Sánchez Márquez et al., 2007, 2010; Unterseher, 2011; Massimo et al., 2015; Zhang and Yao, 2015; Rajamani et al., 2018). While it is recognized that most, if not all, plants contain endophytes, many remain to be studied for their functionality.
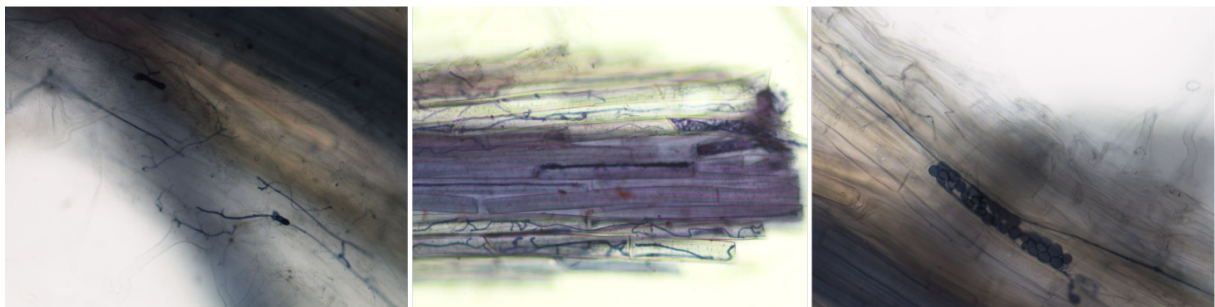
Figure 1.1 – Endophytic fungal structures in inter and intracellular spaces observed by light microscopy in roots of plants.

Fungal endophytes can be classified into four groups: clavicipitaceous endophytes (Class 1), non-clavicipitaceous endophytes that colonize both above and below ground tissues (Class 2), non-clavicipitaceous endophytes that are restricted to above-ground tissues form highly localized infections (Class 3) and non-clavicipitaceous endophytes that are restricted to roots (Class 4) (Figure 1.2) (Rodriguez et al., 2009).

Figure 1.2 – Classes of fungal endophytes and their mode of natural transmission. **Class 1 -** Clavicipitaceous endophytes proliferate within grasses and are transmitted vertically with their seeds; **Class 2 -** Non-clavicipitaceous endophytes that colonize both above and below ground tissues and can be transmitted horizontally and vertically; **Class 3 -** Non-clavicipitaceous endophytes that are highly localized and restricted to above-ground tissues and are transmitted horizontally; **Class 4 -** Non-clavicipitaceous endophytes that are restricted to roots and are transmitted horizontally; **Vertical transmission –** Transmission of fungal endophytes from generation to generation through of infected seeds; **Horizontal transmission –** transmission of fungal endophytes to other associated plants by aire-spores or through movement in soil.

Class 1 endophytes (*Epichloë* endophytes) are phylogenetically related, proliferate within cool and warm season grasses, and are transmitted vertically through their seeds (Figure 1.2) (Bischoff and White, 2005). These endophytes typically spend their entire life cycle within the aerial part of the grass host in a systemic and intercellular association (Rodriguez et al., 2009). Class 1 endophytes might confer benefits to their plant hosts, such as plant biomass increase, improving tolerance to abiotic stress and production of alkaloids, which are toxic for mammals and insects, but these benefits can vary depending on the host and environmental

conditions (Clay and Schardl, 2002). Class 3 endophytes include the hyperdiverse endophytic fungi that are highly localized and restricted to aboveground tissues and they are transmitted horizontally (Figure 1.2) (Rodriguez et al., 2009; Chitnis et al., 2020). Comparatively, Class 2 and 4 endophytes are capable of extensive tissue colonization (Figure 1.2). Although members of both classes are transmitted horizontally, Class 2 endophytes are also transmitted vertically. An important characteristic of Class 2 endophytes is the ability to confer habitat-specific stress tolerance to host plants and non-host plants in extreme habitats (Rodriguez et al., 2008). Dark septate endophytes (DSE) are representative of Class 4 and can be characterized as sterile or conidial. They have dark melanized hyphae and microsclerotia and can play an important role in plant ecophysiology (Porras-Alfaro et al., 2008). DSE endophytes are frequently associated with plants growing in stressful conditions (Knapp et al., 2015).

1.4. Together is better

The fungal endophytic community can be affected by factors associated with the environment and the host plants. Environmental conditions, such as temperature, soil characteristics, geographic localization and vegetation can significantly affect the assembly and distribution pattern of endophytic fungi (Jia et al., 2016). For instance, Glynou et al. (2016) showed a strong influence of the local environment in the root endophytic communities and Dang et al. (2021) found that the plant genotype, the ecological region, and the physicochemical properties of the soil contributed to the differences in endophytic fungal communities in *Glycyrrhiza*. Similarly, plants adapted to stressful environments could be symbiotically associated with endophytic fungi that were better suited to their needs and environmental conditions, resulting in distinctive fungal endophyte communities. For example, plants adapted to saline and dry environments can become symbiotic with fungal endophytes that play an important role in plant adaptation to stress conditions and growth promotion (González-Teuber et al., 2017; Khalmuratova et al., 2021). Therefore, plants can form a symbiotic association with fungal endophytes that can confer protection and habitat-specific adaptation (Rodriguez et al., 2008). In return, plants provide protection, dissemination, spatial structure, and nutrition supply to the microorganism (Schulz and Boyle, 2006; Hardoim et al., 2015).

Collectively, fungal symbiotic associations influence host performance conferring several fitness benefits (Lugtenberg et al., 2016). Thus, there is great interest in the use of microorganisms for crop improvement (Chitnis et al., 2020; Vázquez de Aldana et al., 2021).

The research on the ability of fungal endophytes to mitigate stress in plants may provide new strategies to increase crop productivity, control pests and overcome the impacts of global climate change on agriculture (Pozo et al. 2021).

1.5. Endophytic fungi, an ally against abiotic stress

Abiotic stresses are major environmental conditions that reduce plant growth, productivity and quality. Salinity and drought are two of the main abiotic stress factors that reduce plant productivity. The symbiotic association with fungal endophytes can provide diverse metabolomic and genetic strategies to reduce the impact of abiotic stresses on plants (Lata et al., 2018; Gupta et al., 2020).

Soil salinization is one of the major soil degradation threats occurring in Europe. It happens when water-soluble salts accumulate in the soil to a level that can affect environmental health, agricultural production, and the economy. The effects of salinization can be observed in numerous vital ecological and non-ecological soil functions (Daliakopoulos et al., 2016). Salinity affects physiological and metabolic activities in plants by reducing water uptake, inducing osmotic stress, oxidative stress, ion toxicity, nutritional imbalances, and disruption of membrane ion transport (Munns and Tester, 2008; Zhao et al., 2020). Several fungal endophytes such as *Serendipita indica*, *Fusarium*, *Penicillium* or *Epichloë* have shown the ability to increase the host plant resistance against salinity (Waller et al., 2005; Rodriguez et al., 2008; Khan et al., 2011; Chen et al., 2019). Fungal endophytes can increase the tolerance in their host through different strategies, for example, by increasing the rate of proline accumulation for an osmotic adjustment, as observed in tomato plants and rice inoculated with *Serendipita indica* (Jogawata et al., 2013). In connection with this, *Serendipita indica* mediated Na^+ exclusion in *Arabidopsis* plants (Lanza et al., 2019). Another strategy used by fungal endophytes is to decrease the accumulation of reactive oxygen species (ROS) and so avoid oxidative stress damage. Fungal endophytes are able to increase enzymatic ROS scavengers, as catalase, ascorbate peroxidase, superoxide dismutase, glutathione reductase, dehydroascorbate reductase and monodehydroascorbate reductase (Baltruschat et al., 2008; Zhang et al., 2016). Finally, other strategies can also be used by fungal endophytes to improve the capacity of plants to respond to salt stress such as photosynthesis capacity improvement or the induction of phytohormones (Gupta et al., 2020).

Drought stress occurs when soil water availability is reduced to critical levels that stop the growth and normal functioning of plants (Kapoor et al., 2020). Drought is a

multidimensional stress that causes a wide range of morphophysiological, biochemical and molecular modifications on plants (Farooq et al., 2009). Endophytic fungi can also play a significant role in improving plant tolerance to drought. For example, in drought stress experiments, plants inoculated with fungal endophytes are more stress resistant and exhibit higher photosynthetic efficiency as well as lower production of ROS, which is suggested to be controlled by an endophyte-mediated improvement of the plant antioxidant system (Harman et al., 2019). Another of the underlying mechanisms is that fungal endophytes can improve the osmotic adjustment through the accumulation of metabolically neutral solutes, such as proline (Moghaddam et al., 2021).

1.6. Endophytic fungi, an ally against biotic stress

Plant-fungal endophyte relationships can enhance plant defense against pathogens and herbivores by (i) direct mechanisms through the production of metabolites, mycoparasitism and competing by space and resources with pathogens; and (ii) indirect mechanisms, where the endophytes stimulate systemic plant resistance mediated by phytohormone signaling (Zabalgogeazcoa, 2008; Pieterse et al., 2014; Latz et al., 2018).

Some fungal endophytes species can produce secondary metabolites with antimicrobial activity that inhibit the growth and reduced the severity of plant pathogenic fungi (Mousa et al., 2015; Terhonen et al., 2016). Also, other fungal endophytes can also increase plant resistance against herbivores (Clay and Schardl, 2002). For example, endophytic fungi belonging to the genus *Epichloë* can produce biologically active alkaloids in the host grass that are toxic to insects reducing the damage to the plant (Schardl et al., 2004; 2013). Some fungal endophytes could have mycoparasitic activity affecting directly the pathogen viability by hyphal coiling or penetration, as observed in the experiments of Cao et al. (2009) and Rajani et al. (2020). Endophytes also use competition to prevent pathogens from colonizing host tissue. Through rapid colonization and scavenging of available nutrients, fungal endophytes thereby occupy the niche that could otherwise be used by a pathogenic organism (Rodriguez et al., 2009). However, this mechanism is most likely to occur in combination with other mechanisms (Latz et al., 2018). Furthermore, entomopathogenic and nematophagous fungal endophytes that enter their hosts directly via the cuticle or natural openings, also constitute an important biological control strategy against different insect and nematode pests (Quesada-Moraga et al., 2014).

Indirect antagonism is another mechanism used by fungal endophytes against pathogens and is mediated *via* the host plants. Endophytic symbionts may improve plant resistance and protect host plants against pathogens and insects *via* Induced Systemic Resistance (ISR) and systemic acquired resistance (SAR), mechanisms that primes the plant immune system for resistance against future attacks via jasmonic acid, ethylene and salicylic acid signaling (Pieterse et al., 2014).

1.7. *Epichloë*, the special one

One of the most studied systems of plant-endophyte associations is that between *Epichloë* (Fr.) Tul. & C. Tul. species (Clavicipitaceae, Hypocreales, Ascomycota) and grasses. Fungi of the genus *Epichloë* are common endophytes in association with Pooideae grasses in a variety of environments (Zabalgogeazcoa et al., 1999; 2003; Leinonen et al., 2019), and the association is facultative for plants but obligate for the endophytes (Saikkonen et al., 2004; Leuchtmann et al., 2014). This symbiosis is highly integrated involving a reciprocal use and manipulation of morphology, physiology, and life cycle and history traits of the partners to increase the fitness of the symbionts (Clay and Schardl, 2002; Saikkonen et al., 2016).

A remarkable characteristic of *Epichloë* is that the growth of hyphae is synchronized with the growth of the host grasses, and all plant tissues are colonized by hyphae with the exception of the roots (Christensen et al., 2008). Thus, *Epichloë* grows throughout the intercellular spaces in the aerial tissues of the host plant including inflorescences (Saikkonen et al., 2016). Some species of *Epichloë* are known to be seed-transmitted to the next generation of the host plant, by vertical transmission (Clay and Schardl, 2002; Schardl et al., 2004; Leuchtmann et al., 2014). However, some strictly sexual *Epichloë* species can be transmitted horizontally, as is the case of *Epichloë typhyna,* that is pathogenic during the host plant reproductive phase, developing fungal stromata around plant inflorescences, causing a condition known as choke disease (Zabalgogeazcoa et al., 2008). When this happens, *Epichloë typhyna* is horizontally transmitted to new host plants by ascospores.

One of the main characteristics of *Epichloë* is the capacity to produce several classes of biologically active alkaloids that provide selective benefits to the host plants: ergot alkaloids, indole-diterpenes, lolines and peramine (Vázquez de Aldana et al., 2003, 2010; Schardl et al., 2004). The ergot alkaloids and indole-diterpenes are known for their toxicity to grazing livestock in the form of fescue toxicosis and ryegrass staggers, respectively, and they can also exhibit insecticidal activity (Guerre 2015; Philippe 2016). In contrast, lolines and peramine

exhibit strong insecticidal activity (Schardl et al., 2013), but they are not toxic to mammalian herbivores. The production of alkaloids varies among *Epichloë* species and strains (Vázquez de Aldana et al., 2010; Schardl et al., 2013; Soto-Barajas et al., 2019).

Epichloë species are typically considered plant mutualists. In exchange for hosting the endophyte, the host grass can receive benefits such as increased growth (Saikkonen et al., 2016), as well as increasing resistance and tolerance to a range of biotic and abiotic conditions such as water limitation (Vázquez de Aldana et al. 2013a), flooding (Song et al., 2015), salinity (Wang et al., 2020), low soil nutrients (Zabalgogeazcoa et al., 2006a), pathogens (Li et al., 2020), and heavy metals or herbivory (Fuchs et al., 2017). In addition, the presence of *Epichloë* can increase host performance in competition with other plant species (Vázquez de Aldana et al., 2011, 2013c).

Because of their ability to increase plant growth, control pests, stress tolerance and overall fitness, *Epichloë* strains have been used for commercial uses. For example, Avanex Unique Endophyte Technology is a commercial *Epichloë* that reduces the attractiveness of airports and surrounding areas to birds through taste aversion and post-ingestion feedback as well as an indirect mechanism by deterring many invertebrates, a food source of many bird species (Pennell et al., 2016). On the other hand, MaxQ (*Epichloë coenophiala*) and AR37 (*Epichloë festucae* var. *lolii*) are examples of commercial *Epichloë* strains that provide bio-protective properties to the host plants against insect pests, but they do not cause any fescue toxicosis symptoms (Johnson et al., 2013). Currently, many turfgrass cultivars from different companies are commercialized containing *Epichloë* endophytes in the seeds.

1.8. *Festuca rubra* subsp. *pruinosa*, our target plant, between the rocks and the sea

The genus *Festuca* contains more than 500 species with a worldwide distribution in different habitats, including mountains, and artic and subarctic areas (Inda et al., 2008). *Festuca* is a large genus of perennial grasses that belongs to the family Poaceae. This genus can be generally divided into fine-leaved plants with thin needlelike leaves and broad-leaved plants with straplike leaves (Torrecilla and Catalán, 2002).

Figure 1.3 – Examples of *Festuca* species adaptated to different environments, such as mountains, cliffs and volcanic islands.

Festuca rubra is one of the most representative species of *Festuca*, cool-season grass with commercial interest, used as turf in ornamental and sports grounds. It is a complex of morphologically variable species with a wide ecological amplitude, showing differentiation through adaptations to a range of environmental factors (Rozema et al., 1978; Markgraf–Dannenberg, 1980). The taxonomy of the *F. rubra* complex has been controversial, so a new discussion in this way has been proposed (Saikkonen et al., 2019). It had a complex evolutionary history, partially due to isolation of *F. rubra* populations during the glaciations resulting in a genetic variation (von Cräutlein et al., 2019). Due to its high morphological variability and adaptation to very diverse ecological niches, the *F. rubra* can be classified in different subspecies, e.g., *arctica, arenaria, aucta, fallax, litoralis, mediana, pruinosa, rubra, secunda, vallicola, commutata* and others (Braun et al., 2020; IPNI; 2021). The subspecies *arenaria, litoralis* and *pruinosa* are adapted to maritime habitats (Markgraf–Dannenberg, 1980).

This thesis focused on *Festuca rubra* subs. *pruinosa* (FRP), a perennial grass that inhabits sea cliffs of the Atlantic coasts (Markgraf-Dannenberg, 1980; López-Bedoya and Pérez-Alberti, 2009). This plant grows as a chasmophyte in rock fissures, or in very shallow soils formed on rock crevices with low nutrient availability, and high exposure to sea water spray and desiccating winds (Figure 1.4). Some anatomical characteristics of FRP might contribute to the adaptation to cliffs, for example dense layer of epicuticular wax covering its leaves, stomata enclosed on the adaxial side of c-sectioned leaves and a thickened root

endodermis (Baumeister and Merten, 1981; Ortuñez and de la Fuente, 2010; Martínez Segarra et al., 2017). Additionally, this grass exhibits a robust, dense and compact fibrous root system that permits better anchoring in the crevices and nutrient uptake (Figure 1.4).

Figure 1.4 – *Festuca rubra* subsp. *pruinosa* grows as a chasmophyte in rock fissures with a robust and dense root system.

FRP is a natural host of the fungal endophyte *Epichloë festucae*, which grows intercellularly and asymptomatically in aboveground tissues and is transmitted vertically by infected seeds to their offspring (Zabalgogeazcoa et al., 2006b). An important characteristic of the symbiotic interaction of FRP with *E. festucae* is related to the production of fungal alkaloids (Vázquez de Aldana et al., 2007). The incidence of *E. festucae* in FRP natural populations is relatively high and it is speculated that the cost for harboring this systemic symbiont in this harsh environment could be compensated by mutualism. However, the functions of *E. festucae* in the adaptation of FRP to its natural environment are not fully understood.

Beyond a symbiosis with *E. festucae,* which colonizes aerial organs, FRP roots might be able to maintain a mutualistic interaction with other fungal endophytes. Until date, no study has described the root mycobiota of FRP and its role in the adaptation to the environment.

This study investigated the culturable endophytic mycobiota community on roots of *Festuca rubra* subsp. *pruinosa* and its role in plant adaptation, as well as its effect on other plant species.

1.9. RESEARCH OBJECTIVES

The main purpose of this thesis was to describe the endophytic mycobiome from the host plant *Festuca rubra* subsp. *pruinosa* and to understand its role in the adaptation to salinity conditions as well as to explore its potential attributes for applications as plant growth promoting agent and to promote tolerance to abiotic and biotic stresses in non-host plant such as tomato. With this broad aim, the specific objectives were as follows (Figure 1.5):

I.   To identify the culturable endophytic mycobiota of roots and the prevalence of *Epichloë festucae* in above tissues of *Festuca rubra* subsp. *pruinosa* from natural populations (Chapter 2).

II.   To explore the effect of the foliar endophyte *Epichloë festucae* and selected root endophytes on the performance of *Festuca rubra* subsp. *pruinosa* plants subjected to salinity (Chapter 3).

III.   To monitor gene expression and metabolomic regulation induced by salt and *Epichloë festucae* in *Festuca rubra* subsp. *pruinosa* (Chapter 4).

IV.   To evaluate the effect and compatibility of *Diaporthe* sp. isolated from *Festuca rubra* subsp. *pruinosa* roots on tomato plants and its role in the adaptation to drought stress (Chapter 5).

V.   To test the effectiveness of *Diaporthe* sp. against *Fusarium* wilt on tomato (Chapter 6).
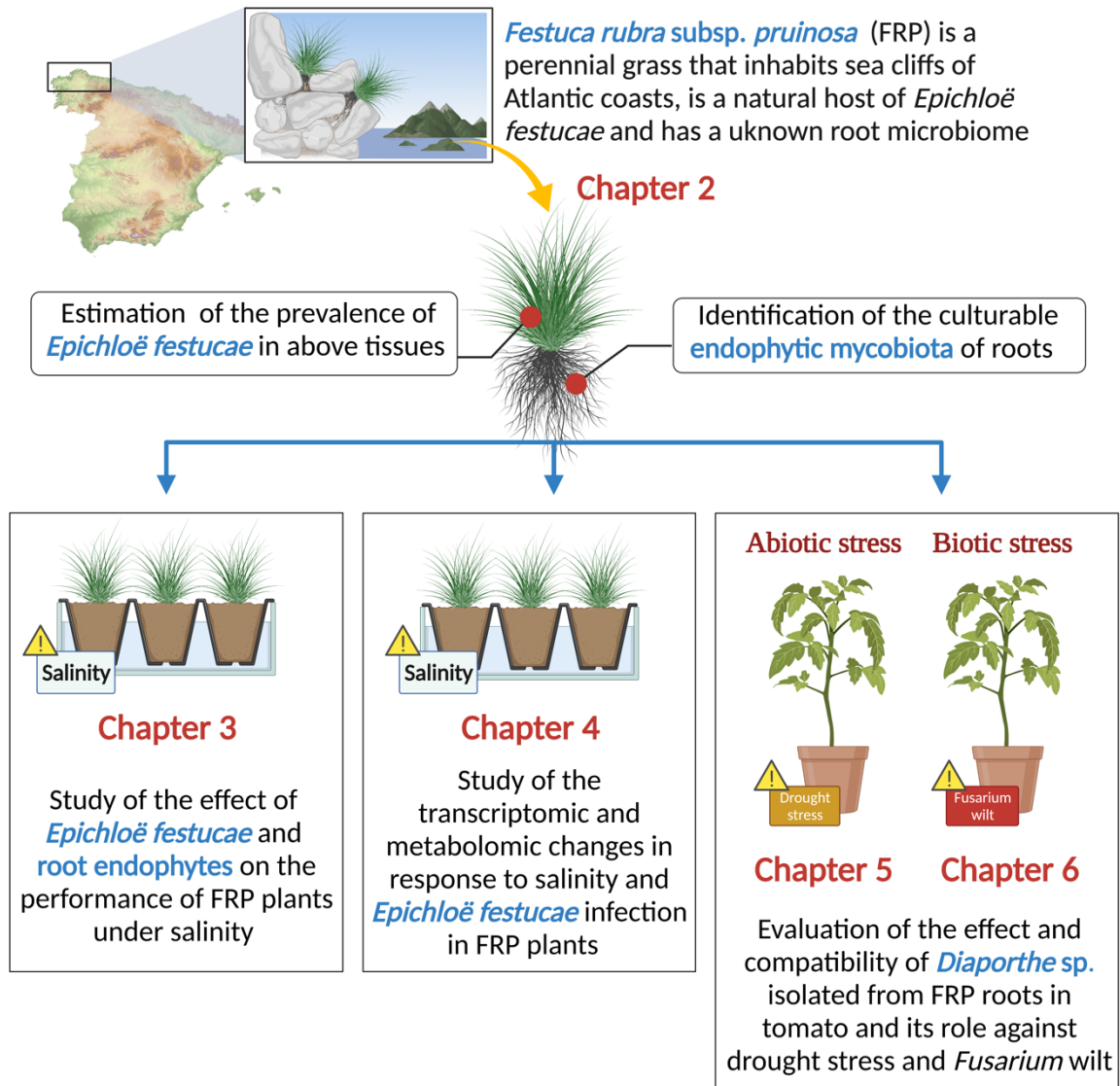
Figure 1.5 – Principal research objectives of the thesis.

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Chapter 2

A survey of culturable fungal endophytes from *Festuca rubra* subsp. *pruinosa*, a grass from marine cliffs, reveals a core microbiome

Abstract

Festuca rubra subsp. *pruinosa* (FRP) is a perennial grass that inhabits sea cliffs of the Atlantic coasts of Europe. In this unhospitable environment plants grow in rock crevices and are exposed to abiotic stress factors such as low nutrient availability, wind, and salinity. FRP is a host of the fungal endophyte *Epichloë festucae*, which colonizes aerial organs, but its root mycobiota is unknown. The culturable endophytic mycobiota of FRP roots was surveyed in a set of 105 plants sampled at five populations in the northern coast of Spain. In total, 135 different fungal taxa were identified, 17 of them occurred in more than 10% of plants and at two or more populations. Seven taxa belonging to *Fusarium*, *Diaporthe*, *Helotiales*, *Drechslera*, *Slopeiomyces* and *Penicillium* appeared to be constituents of the core microbiome of FRP roots because they occurred in more than 20% of the plants analyzed, and at three or more populations. Most fungal strains analyzed (71.8%) were halotolerant. The incidence of *Epichloë festucae* was 65.7 %, but its presence did seem to affect significantly the structure of the core or other root microbiota, when compared to that of plants free of this endophyte. When plants of the grass *Lolium perenne* were inoculated with fungal strains obtained from FRP roots, a *Diaporthe* strain significantly promoted leaf biomass production under normal and saline (200 mM NaCl) watering regimes. These results suggest that the core mycobiome of FRP could have a role in host plant habitat adaptation, and might be useful for the improvement of agricultural grasses.

2.1. INTRODUCTION

The vegetation that inhabits coastal marine cliffs is adapted to environmental conditions that are far from optimal for plant growth and survival. The rock substrate and vertical cliffs makes soil scarce or non-existent. Sea water spray adds salinity to the scenario, and exposure to sea winds favor plant dehydration. Those conditions of low nutrient availability, salinity, and wind exposure can be persistent in sea cliffs, and as a result, sea cliff vegetation is often endemic, reflecting habitat specialization in order to survive under these unhospitable conditions (Doody, 2001; López-Bedoya and Pérez-Alberti, 2009).

Festuca rubra subsp. *pruinosa* (FRP) is a plant species common in cliffs of the Atlantic coasts of Europe (Markgraf-Dannenberg, 1980; López-Bedoya and Pérez-Alberti, 2009). This perennial grass grows as a chasmophyte in rock fissures, or in very shallow soils formed on cliff cavities and slopes. In nature this species rarely occurs away from sea cliffs, where other vegetation predominates, and its salt tolerance is greater than that of other *F. rubra* subspecies adapted to inland habitats (Humpreys, 1982). Some anatomical characteristics might contribute to the adaptation to cliffs of this plant, for instance, the epithet *pruinosa* refers to the apparent epicuticular wax coat that covers its leaves, possibly having a role in preventing water loss (Ortuñez and de la Fuente, 2010; Martínez Sagarra et al., 2017).

In addition to traits inherent to the plant genome, the plant microbiome can also contribute to adaptation. Studies of some plants adapted to high stress habitats revealed that fungal endophytes confer habitat-specific stress tolerance to their hosts, and without these fungal endophytes plant adaptation is reduced in their native habitats (Rodriguez and Redman, 2008). Examples include improved tolerance to biotic and abiotic stress factors such as disease, herbivory, heat, or salinity mediated by endophytic fungi (Clay and Schardl, 2002; Waller et al., 2005; Rodriguez et al., 2008). Some of the endophytes reported in these studies conferred improved stress tolerance to new host species, highlighting the importance that endophytic fungi could have for the improvement of agricultural crops.

Like other subspecies of *Festuca rubra*, FRP plants maintain associations with the fungal endophyte *Epichloë festucae*. This fungus systemically colonizes the stems and leaves of host plants, but not the roots, and it is transmitted vertically to seeds (Leuchtmann et al., 1994; Zabalgogeazcoa et al., 2006). Endophytic *Epichloë* species can have a mutualistic relationship with their hosts, and increased tolerance of symbiotic plants to biotic and abiotic stress factors have been reported to occur in some situations. For example, *Epichloë festucae*

can produce several types of alkaloids that might protect host plants against herbivores (Clay and Schardl, 2002).

In marine cliffs the roots of FRP plants grow in rock fissures or minimal soil, forming a compact fibrous system which holds the plant and captures nutrients. The root mycobiota of FRP is unknown, and some of its components could be useful for the improvement of other plant species of agronomic interest, as it has been demonstrated in other plant-endophyte associations (Rodriguez et al., 2008). Thus, the objectives of this work were: (1) to identify the culturable endophytic mycobiota of *Festuca rubra* subsp. *pruinosa* roots, (2) to determine if the presence of *Epichloë* affects the structure of the root mycobiota, and (3) to test if some FRP root endophytes affect the performance of another grass, *Lolium perenne*, when exposed to salinity.

2.2. MATERIALS AND METHODS

2.2.1. Study sites and plant sampling

Plants of *Festuca rubra* subsp. *pruinosa* (FRP) were collected at five locations in sea cliffs in the North Atlantic coast of Spain. Three locations were in Galicia: Torre de Hércules (TDH), 43°23′09″N 8°24′23″W, Cedeira (CED), 43°40′46″N 8°01′15″W, and Estaca de Bares (EDB), 43°47′25″N 7°41′16″W, and two in Asturias: San Pedro de la Rivera (SPR), 43°34′43″N 6°13′17″W, and Cabo de Peñas (CDP), 43°39′02″N 5°51′00″W. The shortest distance in straight line among these locations is 30 km. The predominant flora in the walls of these sea cliffs mainly consisted of *Festuca rubra* subsp. *pruinosa*, *Armeria* spp. and *Crithmum maritimum*. The climate in the coast of Galicia and Asturias is mild with oceanic influence and abundant rainfall spread over the year; during the 1981–2010 period the mean annual precipitation was 1106 and 1062 mm, and the average annual temperature 13.5 and 13.8 °C in Galicia and Asturias, respectively (AEMET, 2012). In the spring of 2016, a total of 105 FRP plants, about 20 plants per location, were collected. Most plants grew in fissures in the rock, where soil was very scarce or absent. The plants were transported in a refrigerated cooler to the laboratory in Salamanca, and processed for the isolation of fungi from roots the day after they were sampled. Afterward the plants were transplanted to pots with a 1:1 (v:v) mixture of peat and perlite and maintained in a wirehouse outdoors.

2.2.2. Isolation of fungi

To isolate fungi from roots, a sample of about 20 root fragments of 4–5 cm was collected from each plant. Each root sample was surface-disinfected with a solution of 20% commercial bleach (1% active chlorine) containing 0.02% Tween 80 (v:v) for 6 min, followed by treatment with an aqueous solution of 70% ethanol for 30 s. Finally, the roots were rinsed with sterile water and cut into pieces about 5 mm long. Thirty root pieces of each sample were plated in two Petri plates (15 pieces/plate) with potato dextrose agar (PDA) containing 200 mg/L of chloramphenicol. This antibiotic was used to exclude the isolation of endophytic bacteria. A root sample of each of the 105 plants was prepared as outlined above, and kept in the dark at room temperature. As mycelium emerged from a root fragment into the agar, a small piece of the mycelium from the leading edge of the colony was transferred to a new PDA plate and maintained at room temperature. The root fragment and remaining mycelium were taken out of the original plate to avoid overgrowth. The plates with root samples were checked daily for the presence of fungi for about four weeks.

The presence of *Epichloë festucae* on each plant was diagnosed by isolation. Several leaf sheaths were collected from each plant, cut into fragments about 5 mm long, and surface disinfected by immersion in a solution of 20% commercial bleach for 10 min. The fragments were then rinsed with sterile water, and about 15 fragments from each plant were placed in a PDA plate containing 200 mg/l of chloramphenicol. The plates were kept at room temperature, and fungi emerging from leaf fragments during the first 2–5 days were discarded together with its leaf sheath fragment. White *Epichloë* mycelium emerging from the extremes of the leaf fragments about one week after plating was transferred to new PDA plates for further identification.

2.2.3. Identification of fungi

The fungal isolates obtained from roots were first grouped into different morphotypes according to morphological characteristics such as colony color, exudate production, mycelium appearance, and growth rate. One or a few isolates of each morphotype were used for further classification based on rDNA nucleotide sequences. Fungal DNA was extracted from a small amount of mycelium scraped from a PDA culture using the Phire Plant Direct PCR Kit (Thermo Fisher Scientific). A ribosomal DNA region including the internal transcribed spacer 1 (ITS1), 5.8S rDNA, and ITS2 was amplified by PCR using primers ITS1 and ITS4 (White et al., 1990). Amplification conditions were: 98 °C for 5 min, followed by 35 cycles of 98 °C for 5 s, 54 °C

for 5 s, and 72 °C for 20 s; after that the reaction was kept at 72 °C for 1 min. PCR amplicons were cleaned (MSB Spin PCRapace, Stratec biomedical, Germany) and sequenced at the DNA sequencing service of the University of Salamanca (Spain).

All the sequences obtained were grouped into operational taxonomic units (OTU), considering that groups of sequences with a similarity greater than 97% belonged to the same OTU. This clustering operation was done using BlastClust software (NCBI, 2004). Afterward, a sequence representative of each OTU was used to search for similar curated sequences at the UNITE fungal database. A taxonomic identity was assigned to each OTU considering that the species rank of a UNITE database match was accepted when the identity between the OTU and database sequences was greater than 97%, and most UNITE matches corresponded to the same taxon. When the similarity was 95% – 97%, or UNITE matches corresponded to several species of the same genus, only the genus rank was accepted. In other cases, the sequences were assigned to orders or families whenever it was reasonable.

2.2.4. Analysis of root fungal diversity

For each location (referred to as population from here on), species accumulation curves showing the relationship between the number of plants sampled and the number of fungal species obtained, were estimated using the 'specaccum' function and the exact method with the Vegan Package in R (Oksanen et al., 2017). Estimations of the maximum number of fungal species at each population were obtained with the Bootstrap and Chao indexes using EstimateS 9.0 software (Colwell, 2005). Shannon's index of diversity (H') was estimated from the relative abundance of each taxon identified. The distribution of the relative abundance of the fungal species was observed with a rank-abundance curve. The similarity of fungal communities between each pair of populations was estimated using Jaccard's index of similarity (J). It is calculated from the equation J = c/(a + b + c), where 'c' is the number of fungal taxa shared between two populations, 'a' the number of fungal taxa unique to the first population and 'b' the number of fungal taxa unique to the second population (Jaccard, 1912).

2.2.5. Effect of *Epichloë* on root mycobiota

Species richness (number of different root endophyte species per plant) was analyzed with a two-way ANOVA with *Epichloë* presence (E+) or absence (E−) and plant population

(CED, CDP, EDB, SPR, and TDH) as factors. A type III sum of squares was used because the number of E+ and E− plants was unbalanced.

Species accumulation curves and beta diversity index estimations, plus a Canonical Correspondence Analysis (CCA) were made using the Vegan Package in R (Oksanen et al., 2017). Species accumulation curves for E+ and E− plants were estimated using the 'specaccum' function and the exact method. Beta-diversity indexes were estimated using the 'betadiver' function and the z index based on the Arrhenius species-area model (Koleff et al., 2003). Differences in beta diversity among groups were determined by Tukey multiple comparisons. A CCA was made because the gradient length of the detrended correspondence analysis (DCA) was greater than four, which indicated a unimodal response (Lepš and Šmilauer, 2003). Taxa appearing in less than three plants were omitted for this analysis; as a result, 61 taxa remained. A forward selection procedure (ordistep function) was used to determine the subset of explanatory variables (*Epichloë* incidence, population, *Epichloë*: population) explaining most variation in root mycobiome. The statistical power of the analysis was assessed by Monte Carlo permutation tests (n= 999).

2.2.6. Salt tolerance of fungal isolates

A set of 46 fungal strains belonging to 20 of the most abundant genera isolated from FRP roots plus nine *Epichloë festucae* strains were analyzed to determine their salt tolerance *in vitro*. For each fungal strain a 6 mm diameter mycelial disk was placed in the center of 9 cm Petri plates with PDA containing three different concentrations of sodium chloride: 600 mM (equivalent to sea water concentration), 300 mM, and a control without NaCl. For each fungal strain and salt treatment three replicate plates were prepared. All plates were incubated at room temperature in the dark. The colony diameter was measured at two perpendicular axes when colonies in the fastest growing medium reached a diameter of 4–6 cm. The effect of salinity treatments on the radial growth of fungal colonies was assessed by means of a one-way ANOVA, and statistical significance of differences among means using Tukey's test ($p< 0.05$).

2.2.7. Extracellular enzyme activity

In vitro cellulase and amylase activity was analyzed for 43 strains belonging to some of the most abundant taxa. The production of cellulase was assayed using the method described by Sunitha et al. (2013) adapted to PDA plates. For each fungal strain a 6 mm diameter mycelial

disk was placed in the center of a 9 cm. Petri plate and incubated for 5 days at 25± 1 °C in the dark. After incubation the plates were flooded with 0.2% (w/v) aqueous Congo Red, and distained with 1 M NaCl for 15 min. The presence of a clear zone surrounding the colony indicated cellulase activity. Amylase activity was assessed on PDA containing 2% (w/v) soluble starch. After incubation the plates were flooded for 15 min with a solution of 1% (w/v) iodine in 2% (w/v) potassium iodide. A clear zone surrounding the colony indicated amylase activity (Hankin and Anagnostakis, 1975).

2.2.8. Inoculation of *Lolium perenne* plants with root endophytes from FRP

To test whether FRP endophytes affect the growth of the grass *Lolium perenne* under salinity, plants were inoculated with three fungal strains belonging to some of the core taxa from FRP roots. A greenhouse experiment was conducted with a completely randomized design with 14 plant replicates for each fungal strain (*Periconia* S6, *Penicillium* E7, and *Diaporthe* S69) and salinity treatment (0 and 200 mM NaCl). Seeds of *Lolium perenne* cv. Tivoli (DLF, Denmark) were sown in 200 ml plastic pots filled with a substrate composed of seven parts of peat and perlite (1:1) previously sterilized at 80 °C for 24 h, mixed with one part (v:v) of fungal inoculum. The fungal inoculum was a 4-week-old culture of each fungus grown in autoclaved sugar beet pulp. Several seeds were sown in each pot and thinned to four seedlings after emergence. Three weeks after germination, plants were watered with 0 or 200 mM NaCl for three weeks. Plants subject to the salinity treatment were watered with 50 and 100 mM NaCl on the first and third day respectively to avoid salt shock, and the 200 mM concentration was applied from day 5th onward. After three weeks of salt treatment the plants were harvested.

Five replicates of each treatment (salt and fungal strain) were analyzed for K and Na concentration by inductively coupled plasma atomic emission spectroscopy (ICP-OES, Varian 720-ES). Previously, dried plant samples were calcined at 450 °C for 8 h, and ashes dissolved in $HCl:HNO_3:H_2O$ (1:1:8).

A two-way ANOVA was made to determine the effects of salt treatment and fungal strain on shoot biomass, K and Na concentrations, and differences between means were assessed using Tukey's test ($p < 0.05$). The success of the inoculation was determined after the harvest by the reisolation of the inoculated fungi from surface disinfected roots, using the method above explained.

2.3. RESULTS

2.3.1. Endophyte isolation

After plating 3150 root fragments on culture media, a total of 2324 fungal isolates were obtained, ranging from 355 to 578 among populations (Table 2.1). Most isolates emerged in the first 5 days after the placement of the roots on plates. Isolates were obtained from 73.8% of the root fragments plated. All sampled plants harbored fungi in their roots, and on average, 21 isolates were obtained from the roots of each plant.

Epichloë festucae was isolated from leaves of 65.7% of the plants. Its incidence among populations ranged from 20.0 to 100.0% (Table 2.1).

Table 2.1 - Incidence of *Epichloë* and fungal species richness in roots of *Festuca rubra* subsp. *pruinosa* at five populations from marine cliffs in Northern Spain. TDH= Torre de Hércules; CED= Cedeira; EDB= Estaca de Bares; SPR= San Pedro de la Rivera; CDP= Cabo de Peñas.

Population	Number of plants analyzed	Incidence of *Epichloë festucae* (%)	Root mycobiota			
			Number of isolates obtained	Colonization[1]	Number of fungal species	Fungal species per plant
TDH	21	57.1	471	74.8	34	1.62
CED	19	68.4	355	62.3	46	2.42
EDB	22	77.3	473	71.7	47	2.47
CDP	20	20.0	447	74.5	59	2.57
SPR	23	100.0	578	83.8	46	2.19
Total/mean	**105**	**65.7**	**2324**	**73.8**	**135**	**1.29**

[1] Percentage of root pieces from which fungi emerged into growth medium.

2.3.2. Identification of fungal isolates and taxonomic structure

When the isolates of each population were grouped according to morphotypes, the TDH isolates were classified into 177 morphotypes, CED in 142, EDB in 125, SPR in 137, and those from CDP in 107.

Nucleotide sequences were obtained from one or more isolates of each morphotype. As a result, 502 ITS1-5.8S-ITS2 nucleotide sequences were obtained, and those differing in similarity by less than 3% were considered to belong to the same taxon. After this clustering process, 138 different sequences remained. These sequences were used to interrogate the

UNITE sequence database, and as a result 135 fungal taxa were identified (Supplementary Table S2.1). Twenty-three taxa were identified to a species rank, 69 to genus rank and the remaining 43 were assigned to an order, class, family or division (Table 2.2). All the taxa could be assigned to 64 different fungal genera, 96% of them within the Ascomycota. Pleosporales, Hypocreales, and Eurotiales were the most representative orders, in terms of the number of taxa (23, 18, and 10%, respectively). The remaining orders were marginally represented (Figure 2.1). Among plant populations the number of fungal taxa ranged from 34 to 59 (Table 2.1).
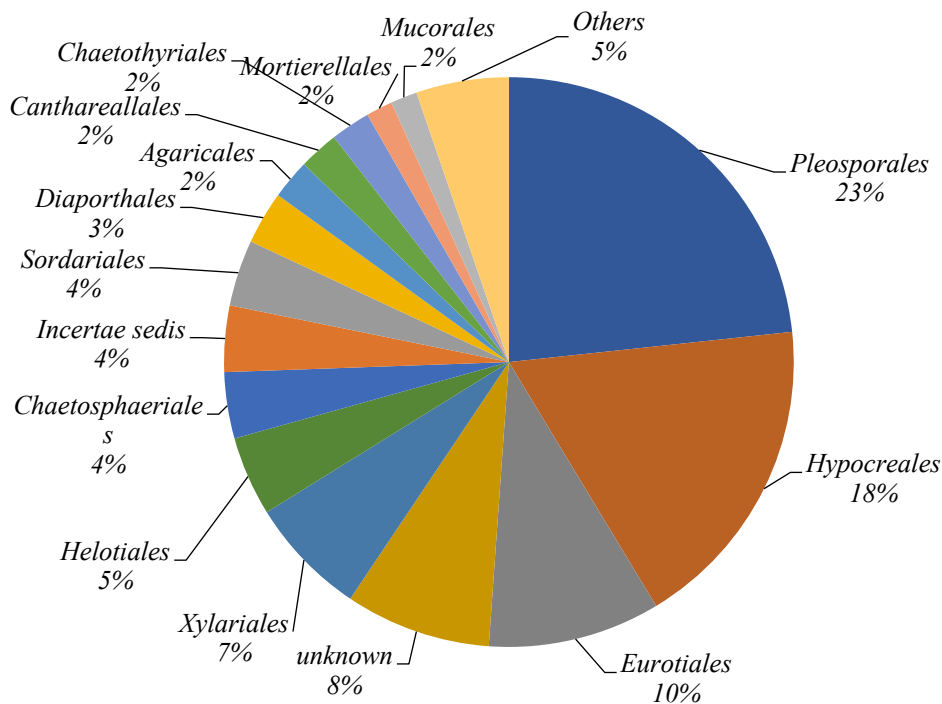
Figure 2.1 - Distribution of fungal taxa from roots of *Festuca rubra* subsp. *pruinosa* plants from marine cliffs in northern Spain according to orders.

The distribution of the taxa according to their incidence can be visualized in the rank-abundance curve shown in Figure 2.2. Seven species occurred in more than 20% of the plants at three or more populations: *Fusarium oxysporum* (57.1%), *Diaporthe* sp. A (54.3%), *Fusarium* sp. A (40.9%), *Helotiales* sp. A (37.1%), *Slopeiomyces cylindrosporus* (27.6%), *Drechslera* sp. (27.6%), and *Penicillium* sp. F (20.0%) (Table 2.2). The identification of several *F. oxysporum* strains was confirmed by Martijn Rep and Maria Constantin (University of Amsterdam) by means of an analysis of their EF1α gene sequence. Because of their relatively high incidence within and among populations, these taxa could be considered as part of the core microbiome of FRP.
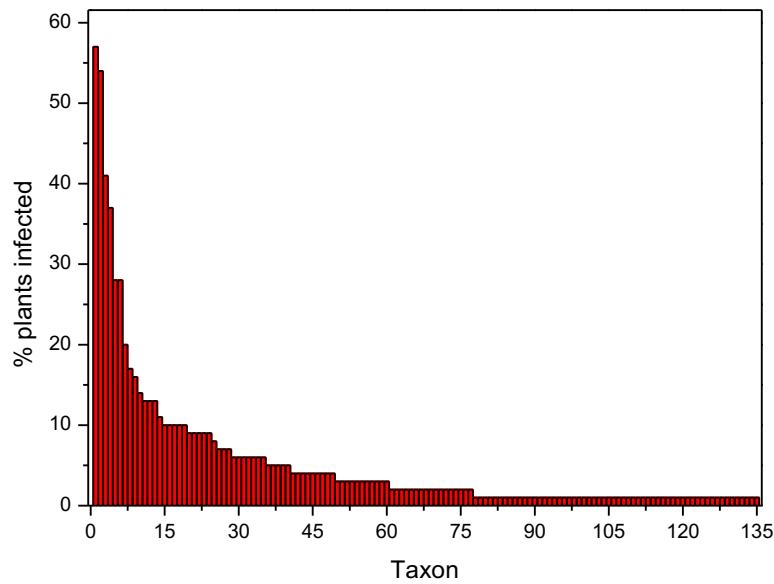
Figure 2.2 - Rank-abundance plot showing the incidence in plants of each taxon identified in roots of *Festuca rubra* subsp. *pruinosa* plants from marine cliffs in northern Spain.

Table 2.2 - Core and abundant fungal species isolated from surface sterilized roots of *Festuca rubra* subsp. *pruinosa* at five populations from northern Spain.

Strain	Taxon	Identity to closest match (%)	ITS sequence accession number	Order	Incidence in plants (%)	Number of Populations
T150	*Fusarium oxysporum*	100	MH578626	Hypocreales	57.14	5
EB4	*Diaporthe* sp. A	100	MH578627	Diaporthales	54.29	5
C29	*Fusarium* sp. A	100	MH626490	Hypocreales	40.95	4
S75	*Helotiales* sp. A	100	MH626491	Helotiales	37.14	5
T105	*Drechslera* sp.	100	MH626492	Pleosporales	27.62	4
S132	*Slopeiomyces cylindrosporus*	100	MH626493	Magnaporthales	27.62	3
T120	*Penicillium* sp. F	100	MH626494	Eurotiales	20.00	5
S7	*Darksidea* sp.	99	MH628220	Pleosporales	17.14	3
T131	*Periconia macrospinosa*	100	MH628221	Pleosporales	16.19	3
T122	*Penicillium* sp. A	100	MH628222	Eurotiales	14.29	4
T16	*Alternaria* sp. A	99	MH628223	Pleosporales	13.33	3
S38	*Fusarium* sp. B	99	MH628224	Hypocreales	13.33	4
C2	*Dactylonectria alcacerensis*	100	MH628225	Hypocreales	13.33	4
E79	*Helotiales* sp. B	100	MH628226	Helotiales	11.43	3
T140	*Alternaria* sp. B	100	MH628227	Pleosporales	10.48	4
E74	*Lachnum* sp. A	99	MH628228	Helotiales	10.48	3
CP17	*Trichoderma* sp. B	100	MH628229	Hypocreales	10.48	2

A second set of relatively abundant taxa were isolated from 10−20% of the plants, and at two or more populations (Table 2.2), these were *Darksidea* sp., *Periconia macrospinosa*, *Penicillium* sp. A, *Alternaria* sp. A, *Fusarium* sp. B, *Dactylonectria alcacerensis*, *Helotiales* sp. B, *Alternaria* sp. B, *Lachnum* sp. A and *Trichoderma* sp. B. The remaining 118 taxa were found in less than 10% of the plants and 58 of them were singletons, occurring in a single plant.

Some of most abundant taxa, like *Darksidea* sp., *Periconia macrospinosa*, *Slopeiomyces cylindrosporus* and *Drechslera* sp., belong to the group of fungi known as dark septate endophytes (DSE). Fungi from the DSE group present some particular morphological characteristics, such as septated and melanized hyphae. These characteristics were observed in hyphae from two strains of *Helotiales* sp. A under the light microscope. Therefore, *Helotiales* sp. A also seems to belong to the DSE.

All populations produced non-asymptotic species accumulation curves, suggesting that increased sampling effort would reveal new fungal species (Figure 2.3). The Chao and Bootstrap estimators of the maximum number of species did not approach a horizontal asymptote, what made them unreliable estimators for this particular case.
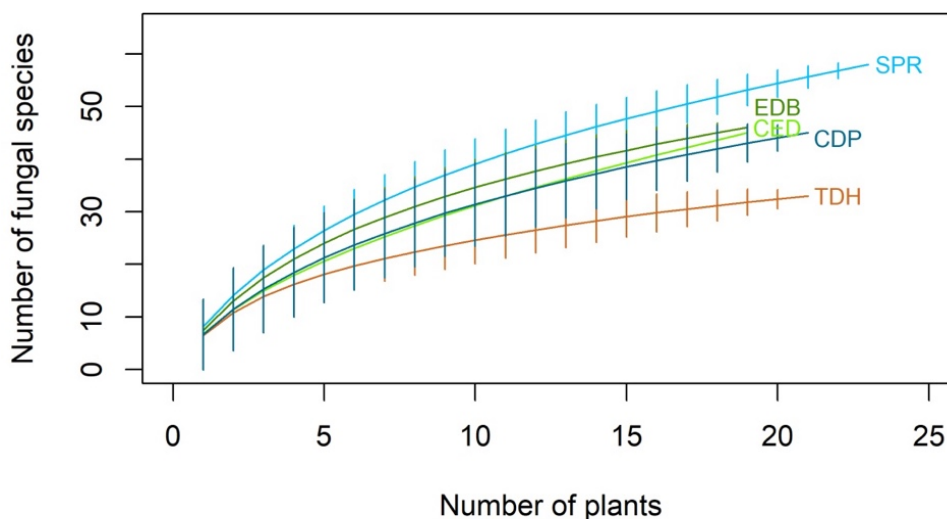
Figure 2.3 - Species accumulation curves for fungal species isolated from roots of *Festuca rubra* subsp. *pruinosa* at five populations from marine cliffs in northern Spain. TDH= Torre de Hércules; CED= Cedeira; EDB= Estaca de Bares; SPR= San Pedro de la Rivera; CDP= Cabo de Peñas.

2.3.3. Effect of *Epichloë* festucae on root endophytic fungal communities

In the set of 105 plants analyzed, 69 were infected by *Epichloë festucae* (E+) and 36 were not (E−). Out of the 135 fungal species identified in all plants, 52 were exclusive of E+ plants, 29 of E− plants, and 54 occurred in both.

The ANOVA showed that neither the presence of *Epichloë* nor population had a significant effect on species richness ($F= 1.999$; $p= 0.276$ and $F= 1.626$; $p= 0.174$ respectively). The beta diversity index showed a similar trend, no significant differences were found between E+ and E− plants ($p= 0.989$) or among populations ($p= 0.377$ for all pairwise comparisons). The values of the Shannon diversity index (H') were relatively high, but similar for E+ and E− plants (Table 2.3).

Table 2.3 - Fungal species richness and diversity in roots of *Festuca rubra* subsp. *pruinosa* plants infected (E+) or not infected (E–) by *Epichloë festucae* at five populations in marine cliffs. TDH= Torre de Hércules; CED= Cedeira; EDB= Estaca de Bares; SPR= San Pedro de la Rivera; CDP= Cabo de Peñas.

Factor		Number of plants analyzed	Species per plant	ß-diversity (Kolleff)	H' Shannon
Epichloë	E+	69	7.19 ±2.63	0.59 ±0.07	4.04
	E−	36	7.08 ±3.11	0.59 ±0.08	3.90
Population	TDH	21	6.52 ±1.91	0.51 ±0.10	3.13
	CED	19	6.84 ±2.59	0.50 ±0.10	3.32
	EDB	22	7.47 ±2.55	0.56 ±0.10	3.48
	CDP	20	6.67 ±3.45	0.55 ±0.10	3.59
	SPR	23	8.17 ±3.04	0.55 ±0.05	3.43

Both E+ and E− plants displayed similar species accumulation curves when the data from all five populations were pooled (Figure 2.4A). The species richness accumulated at 36 plants was 80.93 ± 5.24 for E+ plants and 72.03 ± 1.04 for E− plants. Within each population, we found small differences (both positive and negative) between E+ and E− plants (Figure 2.4B–F).
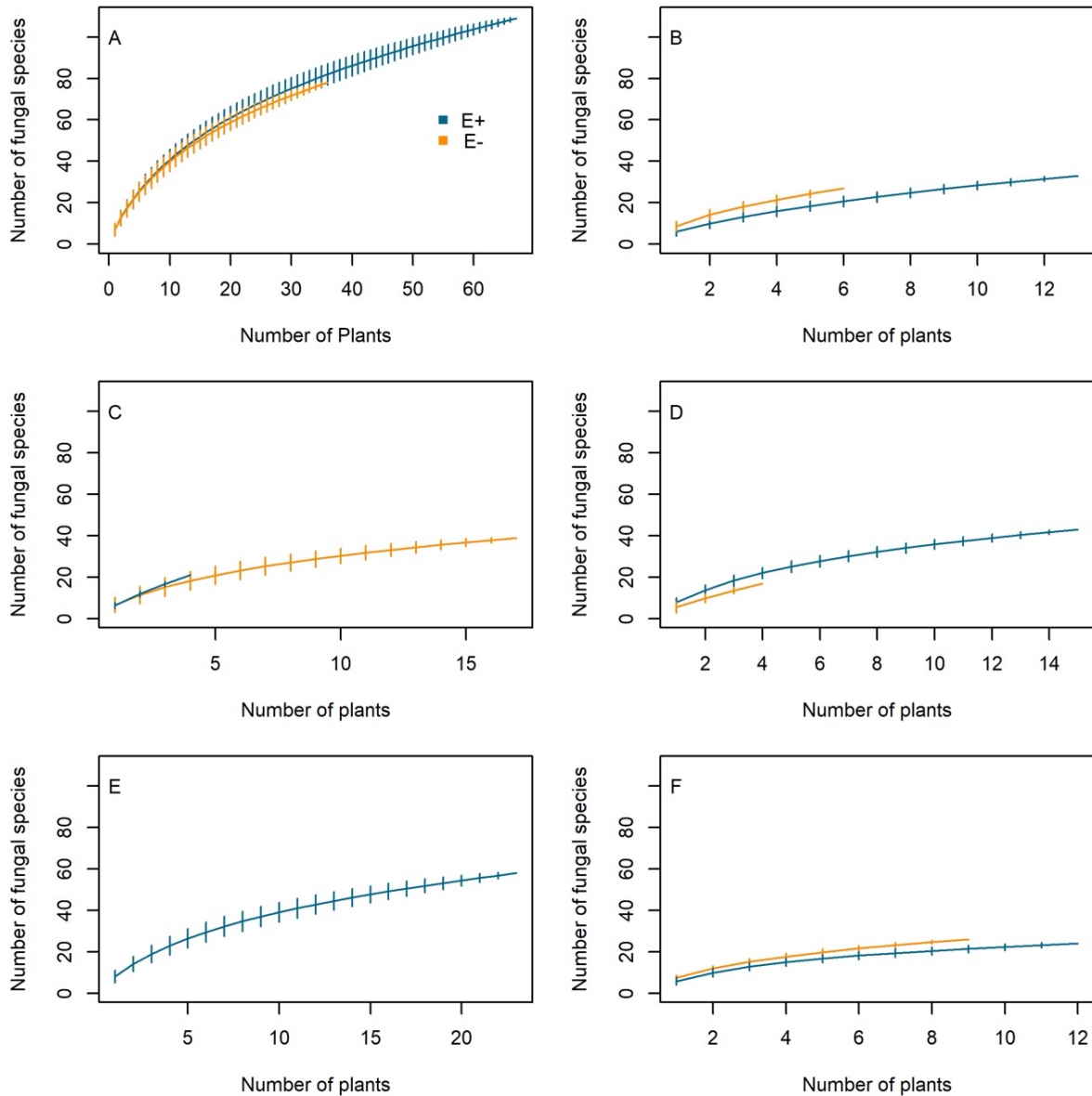
Figure 2.4 - Species accumulation curves of root mycobiota in *Epichloë festucae* infected (E+) and non-infected (E–) plants of *Festuca rubra* subsp. *pruinosa* from five marine cliff populations in northern Spain. **(A)** Whole plant set; **(B)** Cedeira; **(C)** Cabo de Peñas; **(D)** Estaca de Bares; **(E)** San Pedro de la Rivera; **(F)** Torre de Hércules.

The first two axes of the CCA were statistically significant (p= 0.001) and explained 35.18 and 29.36% of the variance. After the forward selection, only the variable population was finally included in the CCA and explained the 5.29% of the variation. The CCA biplot showed no clear separation between E+ and E− plants (Figure 2.5). However, there was a segregation among plant populations: the first axis clustered populations according to regions and separated the Asturian populations (CDP and SPR) from the Galician ones (TDH, CED and EDB); and the second axis segregated both Asturian populations, suggesting that the structure of the root mycobiota of these two populations differ between them and with respect to the Galician populations (Figure 2.5). All the core and the abundant taxa were present in

both E+ and E− plants, although some species were more abundant in E+ (*Slopeiomyces cylindrosporus*) or in E− plants (*Drechslera* sp.) (Figure 2.6).
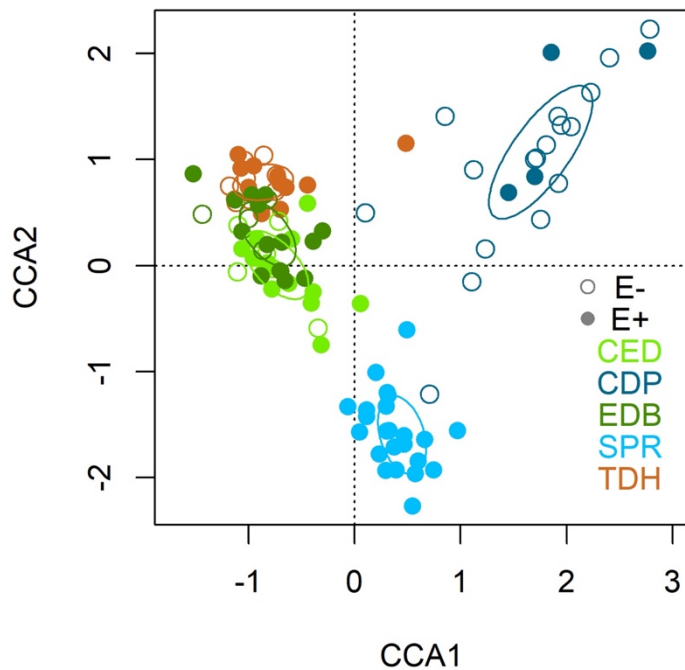
Figure 2.5 - Canonical correspondence analysis (CCA) of the fungal endophyte community composition of roots of *Festuca rubra* subsp. *pruinosa* from marine cliffs according to the presence (E+) or absence (E–) of *Epichloë festucae*, and population (CED= Cedeira; CDP= Cabo de Peñas; EDB= Estaca de Bares; SPR= San Pedro de la Rivera; TDH= Torre de Hércules).
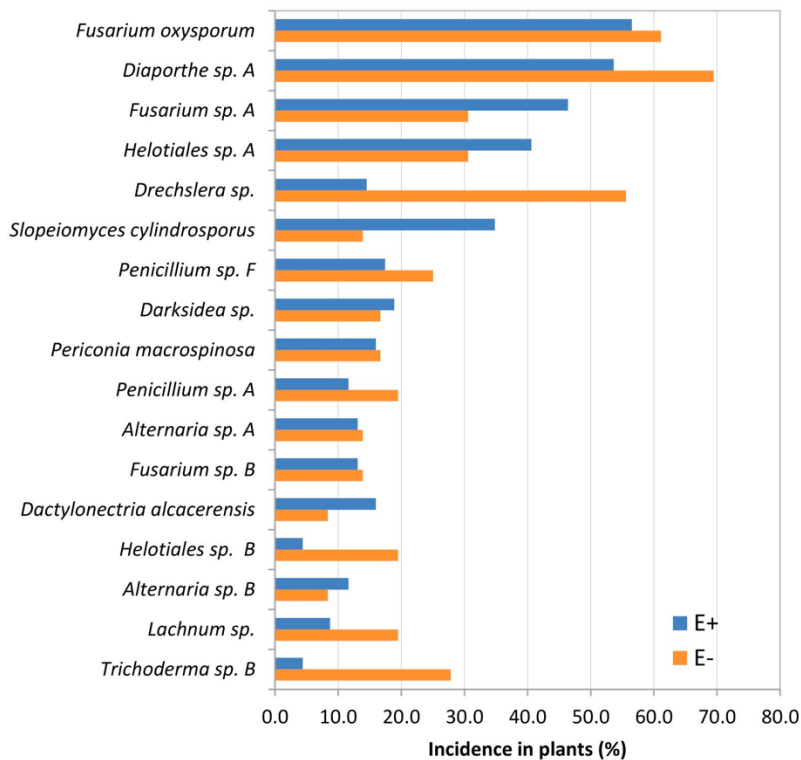
Figure 2.6 - Incidence in plants of *Festuca rubra* subsp. *pruinosa* from marine cliffs infected (E+) and not infected (E–) by *Epichloë festucae* of root species that constitute the core and abundant classes of the culturable mycobiome.

In terms of similarity of the fungal assemblages between pairs of populations, J values were higher between populations from the same region, 0.238 to 0.362 among Galician populations and 0.238 between Asturian populations, than between Galician and Asturian populations, which ranged from 0.095 to 0.193 (Table 2.4).

Table 2.4 - Jaccard index of similarity (blue) and number of fungal species identified in roots of each pair of populations (red) of *Festuca rubra* subsp. *pruinosa* plants from marine cliffs.

Population	TDH	CED	EDB	SPR	CDP
TDH	1.000	0.238	0.362	0.095	0.147
CED	63	1.000	0.300	0.182	0.154
EDB	58	70	1.000	0.193	0.182
SPR	84	88	88	1.000	0.238
CDP	68	78	77	84	1.000

2.3.4. Salt tolerance and enzymatic activity of endophytic fungi

The salt tolerance assay showed that fungal strains had three different types of response in terms of their radial growth. Most strains analyzed (71.8%) were halophilic, showing a statistically significant increase in radial growth in PDA plates containing NaCl respect to the control (Supplementary Table S2.2). The radial growth of 51.5% of these halophilic strains increased at both NaCl concentrations; that of 21.2% increased only in 600 mM NaCl, and that of 27.3% increased only in 300 mM NaCl. All nine *Fusarium* strains and four of the five *Diaporthe* sp. A strains tested were halophilic.

Some strains (6.5%) were halotolerant, not showing a significant difference in radial growth in 300 mM and 600 mM NaCl with respect to the control. Finally, 21.7% of the strains showed a radial growth decrease in culture media containing NaCl and were classified as halosensitive, 80.0% of these strains decreased only in 600mM NaCl, and the remaining 20.0% did it at both salt concentrations. Within taxa like *Diaporthe* sp. A, *Periconia macrospinosa* or *Penicillium* sp. F, some strains had different responses, *i.e.*, *Diaporthe* strain S129 was halophilic and strain S69 halosensitive (Supplementary Table S2.2).

The nine *E. festucae* strains tested were halosensitive, all decreased in radial growth in the 600 mM medium (Table 2.5). Seven of them did not show a significant difference in radial growth with respect to the control at 300 mM NaCl.

Table 2.5 - Radial growth of nine *Epichloë festucae* strains isolated from *Festuca rubra* subsp. *pruinosa* plants from marine cliffs in PDA plates with different NaCl concentrations.

Strain	Radial growth (cm)			Type of response	
	0 mM	300 mM	600 mM		
TDH1	2.12 ab	2.40 b	1.60 a	Halosensitive	
TDH11	1.97 ab	2.42 a	1.82 b	Halosensitive	
CED6	2.67 a	2.17 ab	1.43 b	Halosensitive	
CED12	2.43 a	2.37 a	1.55 b	Halosensitive	
CED10	2.48 a	2.40 a	1.32 b	Halosensitive	
CED1	2.42 a	2.33 a	1.42 b	Halosensitive	
TDH3	2.67 a	2.60 a	1.62 b	Halosensitive	
EDB9	2.37 a	1.25 b	0.67 c	Halosensitive	
EDB11	2.85 a	1.65 b	0.68 c	Halosensitive	

For each row different letters indicate significant differences at $p < 0.05$.

Cellulase and amylase activities were assayed for 43 fungal strains (Table 2.6). Twenty-three of these strains, including all tested strains of *Fusarium oxysporum*, *Penicillium* and *Helotiales* sp. A, showed cellulase activity *in vitro*. In contrast, none of the six *Diaporthe* sp. A strains tested was positive. Amylase activity was detected in only nine strains, including the four *Penicillium* strains tested.

Table 2.6 - Cellulase and amylase activity in fungal strains isolated from roots *Festuca rubra* subsp. *pruinosa* plants from marine cliffs.

ID	Endophyte	Cellulase activity	Amylase activity
T16	*Alternaria* sp. A	−	++
C115	*Alternaria* sp. B	−	+
T90	*Codinaeopsis* sp.	++	−
C2	*Dactylonectria alcacerensis*	−	−
C1	*Darksidea* sp.	+	−
C7	*Darksidea* sp.	+	−
CP36	*Diaporthe* sp. A	−	−
EB4	*Diaporthe* sp. A	−	−
S129	*Diaporthe* sp. A	−	+
S32	*Diaporthe* sp. A	−	−
S69	*Diaporthe* sp. A	−	−
T18	*Diaporthe* sp. A	−	−
CP1	*Drechslera* sp.	−	−
E71	*Drechslera* sp.	−	−
T41	*Drechslera* sp.	−	−
T50	*Drechslera* sp.	−	−
CD8	*Epichloë festucae*	−	−
S13	*Fusarium* sp. A	+	−
T112	*Fusarium* sp. A	+	−
T6	*Fusarium* sp. A	+	−
C70	*Fusarium* sp. B	+	−
S38	*Fusarium* sp. B	+	+
CP3	*Fusarium oxysporum*	++	−

ID	Endophyte	Cellulase activity	Amylase activity
S10	*Fusarium oxysporum*	+	++
SP8	*Fusarium oxysporum*	+	−
T150	*Fusarium oxysporum*	+	−
E79	*Helotiales* sp. B	+++	−
S74	*Lachnum* sp.	−	−
C44	*Helotiales* sp. A	++	−
S75	*Helotiales* sp. A	++	−
T141	*Helotiales* sp. A	++	−
T29	*Helotiales* sp. A	+	−
T3	*Helotiales* sp. A	++	−
T114	*Penicillium* sp. F	++	+++
C13	*Penicillium* sp. A	+	+
E7	*Penicillium* sp. A	+++	+
T59	*Penicillium* sp. A	++	+
S6	*Periconia macrospinosa*	−	−
T131	*Periconia macrospinosa*	−	−
C43	*Slopeiomyces cylindrosporus*	−	−
S5	*Slopeiomyces cylindrosporus*	−	−
T70	*Slopeiomyces cylindrosporus*	−	−
CP17	*Trichoderma* sp. B	++	−

(−) No enzymatic activity; (+) Slight activity; halo <3mm. (++)
Moderate activity; halo <5mm. (+++) High activity; halo >5mm.

2.3.5. Effect of FRP endophytes on growth of *Lolium perenne*

A two-way ANOVA showed a significant effect of salinity ($p= 0.004$; $\bar{X}_{control}= 0.236$ g, $\bar{X}_{NaCl}= 0.192$ g), endophyte inoculated ($p< 0.001$; $\bar{X}_{control}= 0.194$ g, $\bar{X}_{Periconia} = 0.231$ g, $\bar{X}_{Penicillium} = 0.109$, $\bar{X}_{Diaporthe} = 0.321$ g), and their interaction ($p= 0.034$) on dry matter production of *L. perenne*. Plants inoculated with *Diaporthe* S69, a *Diaporthe* sp. A strain, showed a significant increase in biomass production with respect to the uninoculated control plants in both watering treatments: 31.3% in tap water and 48.9% under saline irrigation (Figure 2.7). The plants inoculated with *Periconia* S6 had greater biomass in both watering treatments, but the difference respect to the controls was not significant. In contrast, plants inoculated with *Penicillium* E7 did not show visual symptoms of stress such as dry leaves, but showed a significant decrease in biomass production under the tap water treatment; in the salinity treatment the difference in biomass was not significant with respect to uninoculated plants. In addition, the biomass of plants inoculated with *Penicillium* E7 did not differ between tap water and salinity treatments.
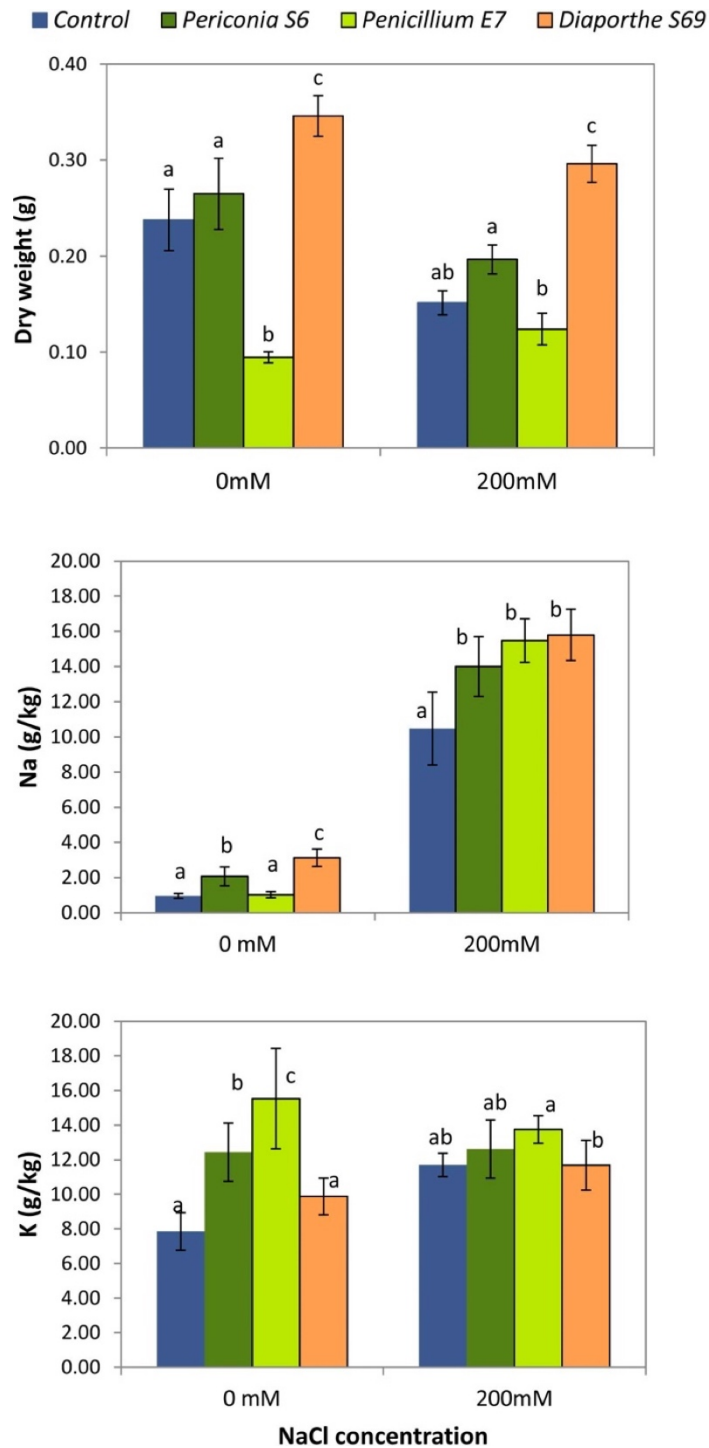
Figure 2.7 - Effect of inoculation with strains *Periconia* S6, *Penicillium* E7 and *Diaporthe* S69, isolated from *Festuca rubra* subsp. *pruinosa*, on dry matter production, and Na and K content of *Lolium perenne* plants watered with 0 mM and 200 mM NaCl. For each NaCl concentration, different letters indicate significantly different means ($p < 0.05$). Values are means ± SE (n=14 for dry weight; n=5 for Na and K).

Sodium was significantly affected by salt ($p < 0.001$), endophyte inoculated ($p < 0.001$) and their interaction ($p = 0.002$). Inoculated plants with *Periconia* S6 and *Diaporthe* S69 strains had greater Na than controls under tap water treatment (Figure 2.7). When plants were salt

irrigated, the increase in Na was greater in plants inoculated with *Penicillium* E7, *Periconia* S6 or *Diaporthe* S69 strains than in control plants. Potassium content was significantly affected by salt ($p= 0.038$), endophyte inoculated ($p< 0.001$) and their interaction ($p= 0.003$). Inoculated plants with E7, S6 or S69 strains had significantly greater K concentration than controls at water treatment (Figure 2.7). At the salt treatment, plants inoculated with *Penicillium* E7 had the greatest K content.

After the harvest, root fragments of *Lolium perenne* were plated on culture media and the fungal isolates obtained were identified through morphological characteristics as the endophytes inoculated into the plants. The reisolation of these fungi indicated their compatibility with *L. perenne* and the success of plant inoculation.

2.4. DISCUSSION

2.4.1. The core microbiome of *Festuca rubra* subsp. *pruinosa*

The roots of *Festuca rubra* subsp. *pruinosa* were found to be a niche containing numerous fungal species, an assemblage of 135 culturable species was identified. This magnitude is not unusual in surveys of the mycobiota of grasses (Sánchez Márquez et al., 2012), but the high incidence of seven species that were present in more than 20% of the plants, and in several populations was remarkable. These species were *Fusarium oxysporum*, *Diaporthe* sp. A, *Fusarium* sp. A, *Helotiales* sp. A, *Drechslera* sp., *Slopeiomyces cylindrosporus*, and *Penicillium* sp. F. In particular, *Fusarium oxysporum* and *Diaporthe* sp. A occurred in more than 50% of the plants, and at all the five populations examined. Those seven fungal species seem to be components of the core microbiome of FRP, because they are shared by a significant number of plants, and occur at different populations (Shade and Handelsman, 2012). It is not common to find a group of fungal species with such high incidence within and among plant populations. Using similar methodology, as well as culture independent methods, no more than two or three species with an incidence greater than 20% were found in surveys of other grasses (Sánchez Márquez et al., 2008, 2010; Ofek-Lalzar et al., 2016; Zhong et al., 2018). In addition, dominant species reported in several taxa of inland grasses, such as *Cladosporium* or *Epicoccum*, were absent from FRP plants (Peláez et al., 1998; Sánchez Márquez et al., 2012; Ofek-Lalzar et al., 2016).

Two of the core taxa of FRP belonged to the genus *Fusarium*. Although this genus is best known due to important pathogens of numerous agricultural species, it is also one of the most commonly isolated genera of endophytes from grasses and other plants (Vázquez de Aldana et al., 2013; Martins et al., 2016; Lofgren et al., 2018). Research on endophytic *Fusarium* has shown that some strains can improve the salinity tolerance of their host plants (Rodriguez and Redman, 2008; Redman et al., 2011). Furthermore, *F. oxysporum* strains obtained from FRP plants in this study were found to protect tomato plants against a pathogenic strain of *F. oxysporum* f. sp. *lycopersici* (Constantin et al., 2017).

The genus *Diaporthe* contains numerous species that behave as endophytes or pathogens, and in some cases as both, depending on the host plant species (Gomes et al., 2013). *Diaporthe* sp. A is a main component of the core microbiome of FRP, and species of this genus have also been reported as dominant components of the microbiome of olive and other plants (Martins et al., 2016; Noriler et al., 2018). Regarding mutualism, *Diaporthe* strains originally isolated from wild plant species promoted the growth of rice and tritordeum (Yang et al., 2015; Zabalgogeazcoa et al., 2018).

Our work revealed that associations between DSE and FRP roots are common in sea cliffs. Some of the core and most abundant taxa, such as *Darksidea* sp., *Periconia macrospinosa*, *Slopeiomyces cylindrosporus* and *Drechslera* sp., were previously reported as DSE in other grasses (Hornby et al., 1977; Knapp et al., 2012, 2015; Siless et al., 2018). In addition, *Helotiales* sp. A also seems to be a DSE because its hyphae had characteristics of this group, and other members of the *Helotiales* (*i.e.*, *Phialocephala fortinii*) are recognized as DSE (Sieber and Grünig, 2013; Ridout et al., 2017). DSE colonize roots of plants communities in different habitats, and some authors hypothesized that these fungi could play an important role in plant adaptation to abiotic stress conditions, especially drought (Porras-Alfaro et al., 2008; Knapp et al., 2015). However, in spite of their abundance in nature, there is still uncertainty about the ecological significance of plant-DSE symbioses (Mandyam and Jumpponen, 2014).

Given the characteristics of the FRP habitat, strains from taxa belonging to the core microbiome of FRP are excellent candidates to test their possible role in host plant adaptation to salinity. Habitat-adapted symbiosis is a phenomenon which occurs when plants establish relationships with symbionts which enhance their adaptation to a particular stress factor present in their habitat (Rodriguez and Redman, 2008). Whether this occurs in the plant-endophyte systems here described would require inoculation of FRP seedlings and evaluation of plant performance parameters under salinity stress. The search for endophytes from the core microbiome of wild plants adapted to unhospitable habitats has produced interesting solutions

for the improvement of stress tolerance on agronomic crops (Redman et al., 2011; Ali et al., 2018).

Because of our research interest in culturable fungi, and the isolation methods used, components of the plant microbiome such as bacteria or non-culturable fungi were not identified in this survey. Members of these groups could have an important role in the adaptation of FRP plants to marine cliffs. For instance, symbioses with arbuscular mycorrizal fungi (AMF) can contribute to plant growth and protection under environmental stress (Lenoir et al., 2016). Symbiotic associations with AMF have been reported for some *Festuca* species (*i.e.*, Dalpé and Aiken, 1998; Santos et al., 2006), but their presence and effects on FRP were not studied, and deserve attention.

In this work, about 72% of the fungal strains from FRP roots were classified as halophilic, their radial growth *in vitro* increased in the presence of NaCl. This category included some species of the core microbiome of FRP, like *Diaporthe* sp. A, *Fusarium oxysporum*, *Fusarium* sp. A, and *Helotiales* sp. A. In contrast, *E. festucae* showed a halosensitive response. The life cycle of this fungus which colonizes the intercellular space of aerial tissues and is seed transmitted, can be completely endophytic. Thus, host plants protect the fungus from the harmful saline environment. However, other fungal species that spend a part of their life cycle outside of their plant hosts might benefit from being halotolerant.

Cellulase or amylase enzymatic activity *in vitro* was detected in some of the core taxa, such as *Fusarium oxysporum*, *Helotiales* sp. A and *Penicillium* sp. F. These enzymes degrade cellulose and starch to soluble sugars such as glucose, cellobiose, and other oligomers which can be readily absorbed by plant roots (Carroll et al., 1983). Considering that FRP plants grow in rock fissures where soil and nutrients are very scarce, fungi with these enzymatic activities could have a role in recycling nutrients from dead roots. However, these two enzymatic activities were not detected in cultures of *Slopeiomyces cylindrosporus*, a fungus with saprobic capability (Hornby et al., 1977), and cellulase activity was absent form *Diaporthe* sp. A strains. This result could be due to non-induction of these enzymes in the culture medium used because both fungal strains grew well as saprobes in a beet pulp medium, rich in carbohydrate and protein, which was used to prepare inoculum for plant inoculations.

2.4.2. Potential of FRP endophytes for plant improvement

Knowledge about the role of endophytic fungi in plant adaptation to salinity stress is important because the world surface of saline soils is increasing, producing economic losses in

crops (Munns and Gilliham, 2015). *Diaporthe* sp. A strain S69 improved the growth of plants of *Lolium perenne*, an important forage grass, in the presence and absence of salinity stress. On average, plants inoculated with *Diaporthe* S69 produced 31% more aerial biomass than the uninoculated controls under normal conditions, and 49% more under salinity stress. Similarly, fungal endophytes such as *Piriformospora indica*, *Fusarium culmorum*, or *Penicillium minioluteum* can alter physiological processes and improve tolerance to salt stress in agricultural crop species (Baltruschat et al., 2008; Khan et al., 2011; Redman et al., 2011).

One of the indirect consequences of salinity is an enrichment of Na and deficiency of K in plant cells, caused by the competition between Na and K, that have similar ionic radii and ion hydration energies (Munns and Tester, 2008). We found that *L. perenne* plants inoculated with *Periconia* S6, *Penicillium* E7 and *Diaporthe* S69 strains accumulated significantly more K in aboveground tissues under the tap water treatment than uninoculated plants; this suggests that an enrichment of Na due to salinity might have been prevented by the increased K content present before the stress. Similar results were observed in grasses inoculated with *Aspergillus aculeatus* (Xie et al., 2017) suggesting that the maintenance of a high level of K may contribute to the alleviation of the negative effect of sodium. A beneficial effect of K accumulation in plants has also been reported for associations with arbuscular mycorrhiza (Langenfeld-Heyser et al., 2007) and *Epichloë* spp. (Chen et al., 2018). It is important to point out that the increase in biomass of *L. perenne* plants inoculated with *Diaporthe* strain S69 occurred not only during salt treatment but also in the tap water treatment. This implies that the fungal effect on improving plant growth was not a specific process induced by salinity. To study the effect of fungal strains on plant parameters which can be altered by endophytes to improve plant performance, such as phytohormones, photosynthetic capacity, nutrient absorption or antioxidant capacity (Baltruschat et al., 2008; Redman et al., 2011; Leitão and Enguita, 2016) is a future objective of our research.

2.4.3. Effect of *Epichloë festucae*, an aboveground tissue endophyte, on root mycobiota

The incidence of *Epichloë festucae* in FRP populations was 65.7%, a value very similar to that of 69% observed in a previous survey that included the same populations from Galicia (Zabalgogeazcoa et al., 2006). The relatively high incidence of *E. festucae* suggests that in an unhospitable habitat like sea cliffs, the costs of harboring a systemic symbiont could be compensated by mutualism. However, endophyte incidences closer to 100% could be expected under such circumstances. Whether natural selection favoring E+ plants, the efficiency of seed

transmission, or a combination of both processes are involved in the prevalence rates of *Epichloë* observed in FRP populations is unknown, and deserves further study. Imperfect seed transmission (<100%) has been reported in other grass–*Epichloë* systems (Gundel et al., 2009). High incidence of *Epichloë festucae* in *Festuca rubra* populations has been reported in semiarid grasslands (70%) (Zabalgogeazcoa et al., 1999), or in the Scottish islands of St. Kilda (80%) (Bazely et al., 1997). In contrast, in Finland only 9 of 49 infected *F. rubra* populations had frequencies greater than 50% (Wali et al., 2007), and no plants harboring *Epichloë* were found in populations from subarctic regions of Canada (Santangelo and Kotanen, 2016).

In some grass-endophyte associations *Epichloë* species could play a key role in salt tolerance. In pot experiments *Epichloë coenophiala* increased the root biomass of tall fescue (*Schedonorus arundinaceous*) (Sabzalian and Mirlohi, 2010), and another *Epichloë* species increased the shoot and root biomass of wild barley (*Hordeum brevisubulatum*) under salinity stress (Song et al., 2015; Chen et al., 2018). In contrast, in FRP plants no significant effect of *Epichloë* on shoot dry weight was detected under salt treatment, although root growth or other parameters that could be affected by the presence of *E. festucae* under salinity were not analyzed (Zabalgogeazcoa et al., 2006). Nevertheless, in a stressful habitat like sea cliffs, environmental pressure on a holobiont might not necessarily affect an individual endophyte, but an assemblage where interactions among the plant host and the eukaryotic and prokaryotic microbiome components might be complex.

The presence of *Epichloë* in aboveground tissues of the host plant can affect underground processes by altering rhizospheric conditions that affect the density and activity of soil microorganisms (Omacini et al., 2012). This may result from endophyte effects on root exudates that can act as chemical attractants or repellents in the rhizosphere (Malinowski et al., 1998). For instance, phenolic compounds are microbial inhibitors, and they increase in roots due to the presence of *Epichloë* (Ponce et al., 2009; Vázquez de Aldana et al., 2011). The effect of *Epichloë* on arbuscular mycorrhizal fungi has been extensively studied, and reduction, promotion, and null effects have been reported (Omacini et al., 2006; Novas et al., 2012; Rojas et al., 2016). Our results indicate that *E. festucae* did not have a clear and significant effect on the composition of the core microbiome or other mycobiota from FRP roots, although changes in the abundance of some species were found. These results are in agreement with other studies where the presence of *Epichloë* did not alter fungal colonization in roots (Vandegrift et al., 2015; Slaughter and McCulley, 2016) or shoots (Zabalgogeazcoa et al., 2013). Nevertheless, Zhong et al. (2018) reported that the presence of *Epichloë* decreased the diversity of root-associated fungi in *Achnatherum inebrians* and changed the community composition.

However, such changes were in fungal orders with an abundance lower than 10%, where the number of isolates of these taxa could be low.

2.5. CONCLUSIONS

In conclusion, this study shows that numerous species of culturable fungi are associated with the roots of *Festuca rubra* subsp. *pruinosa* in its sea cliff habitat. Within this fungal assemblage of 135 species, a set of seven species occurred in a relatively high number of plants and locations, and those seem to be components of the core mycobiome of FRP: *Fusarium oxysporum*, *Diaporthe* sp. A, *Fusarium* sp. A, *Helotiales* sp. A, *Drechslera* sp., *Slopeiomyces cylindrosporus*, and *Penicillium* sp. F. Strains of these species are very promising candidates to study their role in the adaptation of FRP plants to salinity, a characteristic stress factor of their habitat. Furthermore, a *Diaporthe* strain belonging to the core taxa significantly improved the growth of *Lolium perenne* plants under normal and salinity stress conditions, showing the potential of the FRP core microbiome for the improvement of agricultural crops.

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2.7. SUPPLEMENTARY MATERIAL

Supplementary Table S2.1 - Taxonomic identification of fungi isolated from surface sterilized roots of *Festuca rubra* subsp. *pruinosa* at five populations from northern Spain.

Strain	Taxon	Identity to closest match (%)	ITS sequence accession number	Order	Incidence in plants (%)	Number of Populations
T150	*Fusarium oxysporum*	100	MH578626	Hypocreales	57.14	5
EB4	*Diaporthe* sp. A	100	MH578627	Diaporthales	54.29	5
C29	*Fusarium* sp. A	100	MH626490	Hypocreales	40.95	4
S75	*Helotiales* sp. A	100	MH626491	Helotiales	37.14	5
T105	*Drechslera* sp.	100	MH626492	Pleosporales	27.62	4
S132	*Slopeiomyces cylindrosporus*	100	MH626493	Magnaporthales	27.62	3
T120	*Penicillium* sp. F	100	MH626494	Eurotiales	20.00	5
S7	*Darksidea* sp.	99	MH628220	Pleosporales	17.14	3
T131	*Periconia macrospinosa*	100	MH628221	Pleosporales	16.19	3
T122	*Penicillium* sp. A	100	MH628222	Eurotiales	14.29	4
T16	*Alternaria* sp. A	99	MH628223	Pleosporales	13.33	3
S38	*Fusarium* sp. B	99	MH628224	Hypocreales	13.33	4
C2	*Dactylonectria alcacerensis*	100	MH628225	Hypocreales	13.33	4
E79	*Helotiales* sp. B	100	MH628226	Helotiales	11.43	3
T140	*Alternaria* sp. B	100	MH628227	Pleosporales	10.48	4
E74	*Lachnum* sp. A	99	MH628228	Helotiales	10.48	3
CP17	*Trichoderma* sp. B	100	MH628229	Hypocreales	10.48	2
S115	*Alternaria* sp. C	98	MH633916	Pleosporales	9.52	4
T90	*Chaetosphaeriaceae* sp. A	99	MH633917	Chaetosphaeriales	9.52	2
T24	*Agaricales* sp.	100	MH633918	Agaricales	8.57	4
S4	*Plectosphaerella cucumerina*	100	MH633919	Incertae sedis	8.57	3
E18	*Pleosporales* sp. C	100	MH633920	Pleosporales	8.57	3
S8	*Pleosporales* sp. F	100	MH633921	Pleosporales	8.57	2
T107	*Sarocladium strictum*	100	MH633922	Incertae sedis	8.57	3
T174	*Helotiales* sp. C	100	MH633923	Helotiales	7.62	3
S113	*Ceratobasidium* sp. A	100	MH633924	Canthareallales	6.67	2
CP15	*Helotiales* sp. D	99	MH633925	Helotiales	6.67	1
S59	*Pleosporales* sp. B	100	MH633926	Pleosporales	6.67	3
S50	*Alternaria* sp. D	100	MH633927	Pleosporales	5.71	2
E35	*Chaetomium novozelandicum*	99	MH633928	Sordariales	5.71	1
C89	*Fusarium* sp. D	100	MH633929	Hypocreales	5.71	2
E58	*Lachnum* sp. B	99	MH633930	Helotiales	5.71	3
T176	*Marasmius* sp. A	99	MH633931	Agaricales	5.71	3
S100	*Nemania diffusa*	100	MH633932	Xylariales	5.71	2
E20	*Pyrenochaetopsis* sp.	99	MH633933	Pleosporales	5.71	2
C22	*Dichotomopilus* sp. A	99	MH633934	Sordariales	4.76	3
CP69	*Ilyonectria* sp. B	100	MH633935	Hypocreales	4.76	2
S94	*Mortierella* sp. A	100	MH633936	Mortierellales	4.76	2
S33	*Diaporthe* sp. B	100	MH633937	Diaporthales	4.76	1
CP74	*Dichotomopilus* sp. B	99	MH633938	Melanosporales	4.76	1

Strain	Taxon	Identity to closest match (%)	ITS sequence accession number	Order	Incidence in plants (%)	Number of Populations
S104	*Chaetosphaeriales* sp. A	100	MH633939	Chaetosphaeriales	3.81	2
T165	*Clohesyomyces* sp.	99	MH633940	Pleosporales	3.81	1
E17	*Clonostachys* sp.	100	MH633941	Hypocreales	3.81	2
CP23	*Ascomycota* sp. B	93	MH633942	Hypocreales	3.81	1
E14	*Ilyonectria* sp. A	99	MH644805	Hypocreales	3.81	1
T25	*Microdochium* sp. A	99	MH633943	Xylariales	3.81	2
CP56	*Pleosporales* sp. I	92	MH633944	Pleosporales	3.81	1
CP88	*Sordariomycetes* sp.	99	MH633945	unknown	3.81	2
T96	*Trichoderma* sp. A	99	MH633946	Hypocreales	3.81	3
T74	*Absidia* sp.	99	MH633947	Mucolares	2.86	2
S68	*Exophiala pisciphila*	100	MH633948	Chaetothyriales	2.86	3
S78	*Fusarium* sp. G	99	MH633949	Hypocreales	2.86	1
S19	*Fusarium* sp. H	100	MH633950	Hypocreales	2.86	1
CP59	*Marasmius tricolor*	100	MH633951	Agaricales	2.86	1
E2	*Penicillium* sp. E	99	MH633952	Eurotiales	2.86	1
S124	*Penicillium* sp. J	100	MH633953	Eurotiales	2.86	1
S107	*Periconia* sp.	100	MH633954	Pleosporales	2.86	1
S133	*Preussia* sp. B	97	MH633955	Pleosporales	2.86	1
T13	*Thozetella* sp.	99	MH633956	Chaetosphaeriales	2.86	2
T138	*Umbelopsis* sp.	99	MH633863	Mucolares	2.86	2
S87	*Acrocalymna* sp.	100	MH633864	Pleosporales	1.90	2
T39	*Ilyonectria robusta*	100	MH633865	Hypocreales	1.90	1
T143	*Ophiosphaerella* sp. A	99	MH633866	Pleosporales	1.90	2
CP14	*Paraconiothyrium* sp.	100	MH633867	Pleosporales	1.90	1
T111	*Paraphaeosphaeria* sp. A	99	MH633868	Pleosporales	1.90	1
S76	*Paraphaeosphaeria* sp. C	100	MH633869	Pleosporales	1.90	1
E45	*Penicillium* sp. G	98	MH633870	Eurotiales	1.90	1
S116	*Penicillium* sp. I	99	MH633871	Eurotiales	1.90	1
CP81	*Penicillium* sp. K	99	MH633872	Eurotiales	1.90	1
S129	*Diaporthe* sp. C	100	MH633873	Diaporthales	1.90	1
C66	*Diaporthe* sp. D	99	MH633874	Diaporthales	1.90	2
T153	*Pleosporales* sp. A	99	MH633875	Pleosporales	1.90	1
E61	*Clavicipitaceae* sp. A	100	MH633876	Hypocreales	1.90	1
S51	*Ceratobasidium* sp. B	99	MH633877	Canthareallales	1.90	1
CP51	*Talaromyces wortmannii*	100	MH633878	Eurotiales	1.90	1
T129	*Talaromyces* sp. A	100	MH633879	unknown	1.90	1
S102	*Ascomycota* sp. E	100	MH633880	unknown	1.90	1
S46	*Acrostalagmus luteoalbus*	100	MH633881	Hypocreales	0.95	1
C42	*Arthrinium* sp.	100	MH633882	Xylariales	0.95	1
S89	*Aspergillus nomius*	100	MH633883	Eurotiales	0.95	1
CP34	*Aureobasidium pullulans*	100	MH633884	Dothideales	0.95	1
CP11	*Bartalinia* sp.	99	MH633885	Amphisphaeriales	0.95	1
E48	*Boeremia exigua*	99	MH633886	Pleosporales	0.95	1
S80	*Capnodium* sp.	99	MH633887	Capnodiales	0.95	1
C48	*Ceratobasidiaceae* sp. A	99	MH633888	Canthareallales	0.95	1

Strain	Taxon	Identity to closest match (%)	ITS sequence accession number	Order	Incidence in plants (%)	Number of Populations
S72	*Dichotomopilus funicola*	100	MH633889	Sordariales	0.95	1
C33	*Chaetomium* sp. C	99	MH633890	Sordariales	0.95	1
C52	*Chaetosphaeriales* sp. B	95	MH633891	Chaetosphaeriales	0.95	1
S125	*Tolypocladium* sp.	99	MH633892	Hypocreales	0.95	1
T87	*Cryptosporiopsis* sp.	99	MH633893	Helotiales	0.95	1
E49	*Hypocreales* sp.	99	MH633894	Hypocreales	0.95	1
C9	*Cylindrocarpon* sp.	99	MH633895	Hypocreales	0.95	1
S63	*Exophiala* sp.	100	MH633896	Chaetothyriales	0.95	1
T155	*Fusarium* sp. F	100	MH633897	Hypocreales	0.95	1
CP19	*Fusarium* sp. I	99	MH633898	Hypocreales	0.95	1
S127	*Gliomastix murorum*	99	MH633899	Hypocreales	0.95	1
C95	*Xylariales* sp. A	100	MH633900	Xylariales	0.95	1
E38	*Microdochium* sp. C	97	MH644806	Xylariales	0.95	1
C90	*Microdochium* sp. D	99	MH633901	Xylariales	0.95	1
S135	*Mortierella* sp. B	100	MH633902	Mortierellales	0.95	1
T166	*Nemania serpens*	99	MH633903	Xylariales	0.95	1
C54	*Neopestalopsis clavispora*	100	MH633958	Xylariales	0.95	1
S28	*Ophiosphaerella* sp. B	100	MH633959	Pleosporales	0.95	1
CP58	*Paraphaeosphaeria* sp. B	100	MH633960	Pleosporales	0.95	1
S84	*Penicillium* sp. H	100	MH633961	Eurotiales	0.95	1
C16	*Pleosporales* sp. D	99	MH633962	Pleosporales	0.95	1
C5	*Pleosporales* sp. E	97	MH633963	Pleosporales	0.95	1
C104	*Pleosporales* sp. G	98	MH633964	Pleosporales	0.95	1
C63	*Pleosporales* sp. H	99	MH633965	Pleosporales	0.95	1
E70	*Pleosporales* sp. J	99	MH633966	Pleosporales	0.95	1
C8	*Sporormiella australis*	99	MH633967	Pleosporales	0.95	1
S25	*Preussia* sp. A	97	MH633968	Pleosporales	0.95	1
C32	*Acrocalymma vagum*	99	MH633969	Incertae sedis	0.95	1
S110	*Scytalidium* sp.	99	MH633970	Helotiales	0.95	1
S111	*Serendipita vermifera*	98	MH633971	Sebacinales	0.95	1
C56	*Sordaria* sp.	100	MH633972	Sordariales	0.95	1
CP95	*Sordariomycetes* sp. B	100	MH633973	unknown	0.95	1
E6	*Stemphylium vesicarium*	99	MH633974	Pleosporales	0.95	1
CP86	*Talaromyces* sp. B	99	MH633975	Eurotiales	0.95	1
C49	*Trichoderma rossicum*	99	MH633976	Hypocreales	0.95	1
S93	*Trichoderma spirale*	99	MH633977	Hypocreales	0.95	1
T139	*Ascomycota* sp. A	97	MH633978	unknown	0.95	1
C28	*Ascomycota* sp. C	98	MH644807	unknown	0.95	1
S71	*Ascomycota* sp. D	99	MH633979	unknown	0.95	1
S105	*Ascomycota* sp. F	100	MH633980	unknown	0.95	1
E26	*Ascomycota* sp. G	99	MH633981	unknown	0.95	1
C27	*Ascomycota* sp. H	99	MH633982	unknown	0.95	1
E63	*Metarhizium* sp.	100	MH633983	unknown	0.95	1
CP66	*Clavicipitaceae* sp. B	98	MH633984	unknown	0.95	1
C67	*Ascomycota* sp. I	98	MH633985	unknown	0.95	1

| Strain	Taxon	Identity to closest match (%)	ITS sequence accession number	Order	Incidence in plants (%)	Number of Populations
S88	*Mortierella* sp. C	100	MH633986	unknown	0.95	1
E78	*Sordariomycetes* sp. C	99	MH633987	Glomerellales	0.95	1
CP96	*Xylaria* sp.	100	MH633988	Xylariales	0.95	1
CP45	*Xylariaceae* sp.	100	MH633989	Xylariales	0.95	1
E60	*Chaetosphaeriaceae* sp. B	99	MH633990	Chaetosphaeriales	0.95	1

Supplementary Table S2.2 - Radial growth endophytic strains from *Festuca rubra* subsp. *pruinosa roots* grown in PDA plates with different NaCl concentrations. Different letters within each row indicate significant differences at $p < 0.05$.

Strain	Taxon	Radial growth (cm)			Type of response
		0 mM	300 mM	600 mM	
E5	*Alternaria* sp. A	4.52 a	5.82 b	5.62 b	Halophilic
S41	*Alternaria* sp. B	3.83 a	5.58 b	5.05 c	Halophilic
S32	*Diaporthe* sp. A	5.92 a	7.50 b	6.77 c	Halophilic
CP36	*Diaporthe* sp. A	1.83 a	4.98 b	5.43 c	Halophilic
S129	*Diaporthe* sp.C	4.65 a	6.60 b	6.20 b	Halophilic
T95	*Fusarium* sp. A	4.52 a	6.42 b	5.63 c	Halophilic
S13	*Fusarium* sp. A	2.23 a	5.50 b	4.03 c	Halophilic
T112	*Fusarium* sp. A	3.20 a	7.43 b	6.17 c	Halophilic
C70	*Fusarium* sp. B	2.23 a	6.83 b	4.08 c	Halophilic
S38	*Fusarium* sp. B	4.90 a	6.87 b	6.57 b	Halophilic
CP8	*Fusarium oxysporum*	5.53 a	7.12 b	7.25 b	Halophilic
S10	*Fusarium oxysporum*	5.40 a	7.17 b	6.65 c	Halophilic
T150	*Fusarium oxysporum*	5.43 a	7.50 b	7.30 b	Halophilic
C44	*Helotiales* sp. A	4.98 a	6.10 b	6.30 b	Halophilic
E79	*Helotiales* sp. B	4.68 a	7.92 b	7.47 c	Halophilic
T59	*Penicillium* sp. A	3.67 a	7.02 b	6.93 b	Halophilic
CP17	*Trichoderma*	5.70 a	7.33 b	6.58 c	Halophilic
S7	*Darksidea* sp.	2.18 a	2.25 a	3.02 b	Halophilic
T50	*Drechslera* sp.	6.67 a	6.55 a	7.30 b	Halophilic
T6	*Fusarium* sp. A	3.82 a	4.07 a	6.57 b	Halophilic
T114	*Penicillium* sp. F	5.60 a	5.73 a	7.32 b	Halophilic
EB4	*Diaporthe* sp. A	3.03 a	2.47 b	6.43 c	Halophilic
T18	*Diaporthe* sp. A	4.45 a	3.50 b	5.78 c	Halophilic
E7	*Penicillium* sp. A	6.65 a	6.07 b	7.20 c	Halophilic
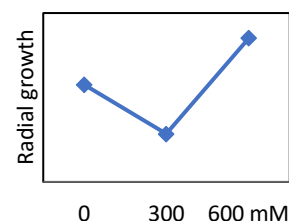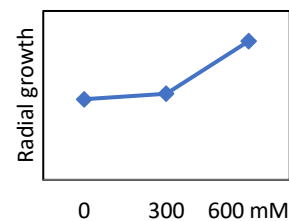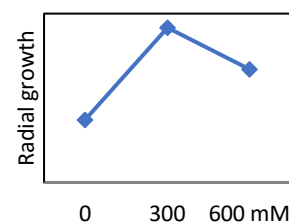
Strain	Taxon	Radial growth (cm)			Type of response	
		0 mM	300 mM	600 mM		
S115	*Alternaria* sp. B	5.20 a	5.80 b	5.52 ab	Halophilic	
T140	*Alternaria* sp. C	5.30 a	6.10 b	5.35 a	Halophilic	
C66	*Diaporthe* sp. A	4.87 a	5.88 b	4.68 a	Halophilic	
C13	*Penicillium* sp. A	4.47 a	4.72 b	4.38 a	Halophilic	
T49	*Periconia macrospinosa*	4.92 a	5.97 b	5.00 a	Halophilic	
T16	*Alternaria* sp. A	4.07 a	5.13 b	3.02 c	Halophilic	
C2	*Dactylonectria alcacerensis*	6.35 a	7.55 b	5.28 c	Halophilic	
T141	*Helotiales* sp. A	5.83 a	6.65 b	5.07 c	Halophilic	
E71	*Penicillium* sp. A	6.43 a	7.05 b	5.72 c	Halophilic	
C1	*Darksidea* sp.	1.33 a	1.83 a	1.57 a	Halotolerant	
E74	*Lachnum* sp.	1.65 a	1.58 a	1.42 a	Halotolerant	
T128	*Helotiales* sp. A	6.35 a	6.13 a	5.80 a	Halotolerant	
S69	*Diaporthe* sp. A	5.12 ab	6.01 a	4.45 b	Halosensitive	
T90	*Codinaeopsis* sp.	5.03 a	5.03 a	3.43 b	Halosensitive	
S75	*Helotiales* sp. A	6.23 a	6.08 a	4.82 b	Halosensitive	
E54	*Penicillium* sp. F	5.48 a	5.71 a	5.03 b	Halosensitive	
T131	*Periconia macrospinosa*	6.77 a	7.25 a	6.00 b	Halosensitive	
S96	*Trichoderma* sp. B	6.15 a	6.23 a	4.66 b	Halosensitive	
S5	*Slopeiomyces cylindrosporus*	7.50 a	7.25 a	1.18 b	Halosensitive	
T70	*Slopeiomyces cylindrosporus*	3.62 a	3.78 a	2.42 b	Halosensitive	
T41	*Drechslera* sp.	6.17 a	5.87 b	5.17 b	Halosensitive	
C43	*Slopeiomyces cylindrosporus*	4.18 a	3.12 b	2.68 c	Halosensitive	

Chapter 3

The role of fungal microbiome components in the adaptation to salinity of *Festuca rubra* subsp. *pruinosa*

Abstract

Festuca rubra subsp. *pruinosa* is a perennial grass that inhabits sea cliffs, a habitat where salinity and low nutrient availability occur. These plants have a rich fungal microbiome, and particularly common are their associations with *Epichloë festucae* in aboveground tissues, and with *Fusarium oxysporum* and *Periconia macrospinosa* in roots. In this study, we hypothesized that these fungi could affect the performance of *Festuca rubra* plants under salinity, being important complements for plant habitat adaptation. Two lines of *Festuca rubra*, each one consisting of *Epichloë* infected and *Epichloë* free clones were inoculated with the root endophytes (*Fusarium oxysporum* and *Periconia macrospinosa*) and subjected to a salinity treatment. Under salinity, plants symbiotic with *Epichloë* had lower Na^+ content than non-symbiotic plants, but this effect was not translated into plant growth. *Periconia macrospinosa* promoted leaf and root growth in the presence and absence of salinity, and *Fusarium oxysporum* promoted leaf and root growth in the presence and absence of salinity, plus a decrease in leaf Na^+ content under salinity. The growth responses could be due to functions related to improved nutrient acquisition, while the reduction of Na^+ content might be associated with salinity tolerance and plant survival in the long term. Each of these three components of the *Festuca rubra* core mycobiome contributed with different functions, which are beneficial and complementary for plant adaptation to its habitat in sea cliffs. Although our results do not support an obvious role of *Epichloë* itself in FRP salt tolerance, there is evidence that *Epichloë* can interact with root endophytes, affecting host plant performance.

3.1. INTRODUCTION

Festuca rubra is a perennial grass that occurs in very diverse ecological niches (Markgraf–Dannenberg, 1980; Inda et al., 2008). In addition to its value as a forage, commercial cultivars of this species are used for ornamental and sports lawns (Braun et al., 2020). Within *Festuca rubra* several subspecies have been defined and three of them, *litoralis*, *pruinosa* and *arenaria*, occur in maritime habitats such as salt marshes, sea cliffs, or coastal sands (Markgraf–Dannenberg, 1980). As a result of habitat adaptation, maritime subspecies are more tolerant to salinity than inland subspecies (Hannon and Bradshaw, 1968; Rozema, 1978). *Festuca rubra* subsp. *pruinosa* (FRP) inhabits sea cliffs of the Atlantic coasts of Europe (Figure 3.1) (Markgraf-Dannenberg, 1980). This grass often grows as a chasmophyte in rock crevices with low nutrient availability and high exposure to sea water spray and desiccating winds. Some structural traits seemingly associated with salt tolerance in FRP are a dense layer of epicuticular wax which covers its leaves, stomata enclosed on the adaxial side of c−sectioned leaves, together with a thickened root endodermis in comparison to inland fescues (Baumeister and Merten, 1981; Ortuñez and de la Fuente, 2010; Martínez Segarra et al., 2017).

Figure 3.1 - *Festuca rubra* subsp. *pruinosa* inhabits rocky sea cliffs of the Atlantic coasts of Europe (left). Plants often grow in rock fissures where soil is absent, and are very exposed to wind and saline sea spray (right).

In addition to the plant traits that may favor tolerance to salinity and water loss, or improve nutrient absorption in a suboptimal environment, the plant microbiome can also provide auxiliary functions that facilitate the habitat adaptation of holobionts (*i.e.*, the host plant and its microbiota) (Rodriguez et al., 2008; Vandenkoornhuyse et al., 2015; Trivedi et al., 2020). In a previous study, 135 different fungal taxa were identified as culturable components of the fungal mycobiome of FRP roots (Chapter 2, Pereira et al., 2019). *Fusarium*

oxysporum was the most abundant taxon, being found at all populations sampled and in 57% of the plants analyzed. *Periconia macrospinosa,* a dark septate endophyte (DSE), was also an abundant component of the root microbiome, and it was found in 16% of the plants. In addition, aerial tissues of 66% of the FRP plants were colonized by the fungal endophyte *Epichloë festucae*. As possible components of the core microbiome of FRP, we here hypothesize that these fungi (*F. oxysporum, P. macrospinosa* and *E. festucae*) could provide functions related to plant adaptation to the sea cliff environment.

Epichloë festucae asymptomatically colonizes the intercellular space of stem and leaf tissues of *F. rubra* and other grasses, and is vertically transmitted to seeds (Clay and Schardl, 2002; Leuchtmann et al., 2014). This fungal endophyte might produce antiherbivore secondary metabolites such as the bioactive alkaloid ergovaline in symbiotic FRP plants (Vázquez de Aldana et al., 2007). Although *Epichloë* endophytes are not present in plant roots, their presence in aboveground plant tissues can affect several belowground processes (Omacini et al., 2012; Rojas et al., 2016). *Epichloë* is one of the best known taxa of endophytic fungi in aboveground tissues, but for most components of root mycobiomes their functions as plant symbionts, as well as their life cycles, are unknown (Pozo et al., 2021).

Soil salinity inhibits plant growth and development by reducing water uptake, and also induces cytotoxicity due to excess of Na^+ ions, oxidative stress due to the generation of reactive oxygen species, and nutritional imbalance (Munns and Tester, 2008; Zhao et al., 2020). Plants, particularly halophytes, have mechanisms involved in adaptation to salinity, like Na^+ exclusion, Na^+ intracellular accumulation and osmoregulation, or alteration of the level of secondary metabolites, including phenolic compounds and their antioxidant capacity (Munns and Tester, 2008; Waśkiewicz et al., 2013; van Zelm et al., 2020; Zhao et al., 2020). In addition, there is compelling evidence of fungal microbiome components contributing to plant adaptation to salinity. For example, some strains of *Epichloë*, *Diaporthe*, *Piriformospora*, *Penicillium, Fusarium*, and DSE have been reported to improve plant growth under salinity (Waller et al., 2005; Rodriguez et al., 2008; Molina-Montenegro et al., 2018; Pereira et al., 2019; Wang et al., 2020, González Mateu et al., 2020).

The main purpose of this study was to explore the effect of the foliar endophyte *E. festucae* and the root endophytes *F. oxysporum* and *P. macrospinosa* on the performance of FRP plants subjected to salinity. We present data supporting that these microbiome components could be involved in the host plant adaptation to its maritime habitat.

3.2. MATERIALS AND METHODS

3.2.1. Plant and fungal material

Two near isogenic lines of FRP (TH12 and EB15) were used to test the effects of *E. festucae* and two root endophytes on plant performance. Each line consisted of a unique plant genotype infected (E+) or not infected (E−) by a unique *E. festucae* genotype. Each line was generated from a single E+ FRP plant originally collected in the coast of Galicia (Figure 3.1) (Chapter 2; Pereira et al., 2019). This plant was split into several clones that were transplanted to 200 ml pots containing a 1:1 (v:v) mixture of peat and perlite. To obtain E− plants, one half of the E+ clones were treated with the systemic fungicide propiconazole to eliminate the fungus (Zabalgogeazcoa et al., 2006a). Six doses of 400 μg of propiconazole were applied to each plant: the first, fourth, fifth and sixth doses were applied to the soil, and the second and third were foliar applications. Fungicide treatments were spaced by five days for the first three treatments and by ten days for the last three soil applications. Four weeks after the last dose, newly formed ramets from each clone were obtained, and their E+ or E− status was verified by direct isolation of *Epichloë festucae* from surface-disinfected leaf sheaths (Florea et al. 2015). Two different near isogenic lines of FRP were used because different *Epichloë*/grass genotypes might differ in terms of stress responses, nutrient accumulation, or alkaloid content (Cheplick et al., 2000; Zabalgogeazcoa et al., 2006a; Vázquez de Aldana et al., 2020b). Only one root endophyte was inoculated into each *Festuca* line because of limited plant clone availability.

The fungal strains *Fusarium oxysporum* T48 and *Periconia macrospinosa* T131 were originally isolated as endophytes from surface-disinfected roots of asymptomatic FRP plants collected in natural populations in the northern coast of Galicia, Spain (Chapter 2, Pereira et al., 2019). These strains were selected because they belonged to two of the most abundant taxa from the culturable fungal microbiome of roots from apparently healthy FRP plants (Chapter 2, Pereira et al., 2019). Thus, we assumed that these microbiome components were non-pathogenic and could have a role in holobiont adaptation. In addition, *Fusarium oxysporum* strains from FRP are non-pathogenic on tomato plants, as shown by Constantin et al. (2020).

3.2.2. Effect of *Epichloë* and root endophytes on plant growth under salinity

To determine the effect of *Fusarium oxysporum* T48 and *Periconia macrospinosa* T131 (onwards *Fusarium oxysporum* and *Periconia macrospinosa*) on the growth of E+ and E− FRP

plants, a greenhouse experiment was designed. Plants of the line EB15 were inoculated with *F. oxysporum*, and those of the line TH12 with *P. macrospinosa*. Each clone to be inoculated with a root endophyte was transplanted to a 200 ml pot containing a substrate composed of seven parts (v:v) of a mixture of peat and perlite (1:1 v/v) previously treated at 80 °C for 24 h, and one part of fungal inoculum prepared in a beet pulp medium for four weeks (Vázquez de Aldana et al., 2020a). Non inoculated clones were transplanted to pots containing only the peat and perlite substrate. A three-factor experiment was carried out for each line, with *Epichloë* infection (E+ or E−), root endophyte inoculation with *F. oxysporum* or *P. macrospinosa* (uninoculated or inoculated), and salinity (watering with 0 or 600 mM NaCl), with five plant replicates per treatment. Plants subjected to the salinity treatment were watered with 200 mM NaCl on the first day to avoid salt shock and with 600 mM NaCl afterwards for five weeks. After this time, the whole plants were harvested, roots were carefully washed with tap water, and some pieces from each plant were kept for observation by optical microscopy. The plants were lyophilized, and their dry biomass was recorded. The aboveground parts, which consisted of leaves and leaf sheaths, were ground and used for chemical analysis.

To check for the presence of *F. oxysporum* or *P. macrospinosa* in roots of all inoculated plants, fresh root fragments were cleared in 5% KOH at 90 ºC for 15 min, neutralized with approximately three volumes of 1% HCl at 20 ºC overnight, stained with trypan blue (Berthelot et al., 2016) and visualized by light microscopy.

3.2.3. Sodium, potassium and proline content

To estimate the concentration of mineral elements, leaf samples (five replicates of each treatment) were calcined at 450 ºC for 8 h and ashes were dissolved in $HCl:HNO_3:H_2O$ (1:1:8). Na and K contents were analyzed by inductively coupled plasma atomic emission spectroscopy (ICP-OES) in a Varian 720-ES (Agilent, USA) spectrometer.

Leaf proline content was quantified in three plant replicates of each treatment using the spectrophotometric method described by Shabnam et al. (2016), adapted to 96-well plates in our laboratory. Approximately 15 mg of plant material were homogenized in 500 µl of 3% aqueous 5-sulfosalicylic acid and kept for 10 min in ice. The mixture was centrifuged at 10 ºC and 16,000 *g* for 10 min, and the supernatant was mixed with 250 µl of glacial acetic and 500 µl of ninhydrin reagent. Then, the mixture was heated at 99 °C for 40 min, and immediately cooled in ice. The mixture was centrifuged and an aliquot of 200 µl transferred to a 96-well

plate where the absorbance was measured at 513 nm in a FLUOStar Omega plate reader (BMG Labtech, Germany). L(−) proline (Acrós Organics) was used as standard for quantification.

3.2.4. Ferric Reducing Antioxidant Potential (FRAP) assay

The total antioxidant capacity was determined in leaves of five replicates of each treatment using the ferric ion reducing antioxidant power (FRAP) method (Benzie and Strain, 1996). This method is based on the reduction of the colorless [Fe(III)−,4,6-tri(2-pyridyl)-*s*-triazine)$_2$]$^{3+}$ complex, abbreviated as Fe(III)−TPTZ, to the blue-colored Fe(II)−TPTZ complex, formed by the action of electron donating antioxidants at low pH. The FRAP reagent was prepared by mixing 300 mM acetate buffer pH= 3.6, a solution of 10 mM TPTZ in 40 mM HCl, and 20.35 mM FeCl$_3$ at a volume ratio of 10:1:1. Five mg of each plant sample were extracted in 700 μl of 50% aqueous acetone for 30 min in an ultrasound bath at 8 ºC. The mixture was centrifuged and transferred to a 96-well plate where 8 μl of sample, 8 μl of phosphate buffer saline (PBS), and 200 μl of FRAP reagent were added to each well. The absorbance was measured at 593 nm after 30 min in a FLUOStar Omega (BMG Labtech, Germany). A standard curve was prepared using different concentrations of 6-hydroxy-2,5,7,8-tetramethylchroman−2−carboxylic acid (Trolox). The TPTZ solution was freshly prepared before use. The results were expressed as μmol trolox equivalent per gram of dry weight.

3.2.5. Phenolic compounds content

The content of total phenolic compounds in leaf samples was determined spectrophotometrically according to the Folin-Ciocalteu method (Ainsworth and Gillespie, 2007). For the analyses, five replicates of each treatment were used. A 100 μl aliquot of acetone extract of each sample, prepared as previously described for the FRAP assay, was mixed with 500 μl of Folin−Ciocalteu reagent (Scharlab Chemie SA). After 5 min, a volume of 400 μl of a 700 mM Na$_2$CO$_3$ solution was added. The mixture was incubated for 60 min and the absorbance at 765 nm was measured in a 96−well plate in a FLUOStar Omega (BMG Labtech, Germany). Gallic acid was used as a reference standard, and the results were expressed as μmol gallic acid equivalent per gram of dry weight.

3.2.6. Ergovaline content

The *Epichloë* alkaloid ergovaline was analyzed in leaf samples of E+ plants (three replicates of each treatment) by HPLC following a modification of the methods described by Hill et al. (1993) and Yue et al. (2000a). Extraction was conducted in 0.5 g of plant material, adding 10 ml of chloroform, 0.5 ml of a methanolic solution 5 mM NaOH, and an internal standard of ergotamine (Sigma-Aldrich). The mixture was placed on an orbital shaker at 100 rpm for 120 min, paper-filtered (Filter Lab 1240), washed with 3.0 ml of chloroform and then passed through an ergosil–HL silica gel (500 mg, Analtech) column preconditioned with 5.0 ml of chloroform. To eliminate pigments, a solution of 5.0 ml chloroform:acetone (75:25 v/v) was passed through the column, and the sample was eluted with 3.0 ml of methanol, and dried under a nitrogen stream. The residue was dissolved in 1.0 ml of methanol and filtered through a 0.45 μm nylon disk. Ergovaline quantification was performed in a Waters 2695 HPLC system, with a C18 column (150×4.6 mm; 2.7 μm; Agilent Poroshell) and a fluorescence detector (Waters 2475). The excitation and emission wavelengths were 250 nm and 420 nm, respectively. The mobile phase was composed by acetonitrile (phase A) and 0.01 M ammonium acetate (phase B) in a linear gradient, at 0.6 ml/min, as follows: 30% A for 18 min; 45% A for 2 min; 85% A for 2 min; 100% A for 5 min; 100% A for 3 min. The ergovaline standard was purchased from Forrest Smith (Auburn University, USA).

3.2.7. *In vitro* dual culture interaction

A dual-culture assay was made to analyze *in vitro* interactions between *Epichloë festucae* and both root endophytes. The *E. festucae* strain from E+ plants of the line TH12 was co-cultured with *P. macrospinosa*, and the *E. festucae* strain from EB15 plants with *F. oxysporum*. Mycelial discs (6 mm diameter) from PDA cultures of each fungus were placed 4 cm apart on the surface of 9 cm Petri dishes containing PDA medium. Controls consisted of agar plates containing two disks of the same strain. Five replicates of each combination were incubated at room temperature in the dark. The evolution of the radii of the interacting strains was measured for several days. Interactions between strains were assessed based on the inhibition or non-inhibition of their mycelial growth.

3.2.8. Statistical analyses

The effects of *Epichloë festucae*, root endophyte inoculation, and salinity on biomass, Na^+ and K^+ content, proline, total phenolic compounds and antioxidant activity were analyzed by means of a three−way ANOVA. For ergovaline concentration in E+ plants, the effects of root endophyte inoculation and salt treatment were analyzed with a two-way ANOVA. The data sets were evaluated for the statistical assumptions of the ANOVA with the Shapiro-Wilk normality test and Brown−Forsythe equal variance test. Differences between means of estimated effects of significant factors and their interaction were evaluated using Tukey's test ($p < 0.05$). All statistical analyses were performed with SigmaPlot v.14.

3.3. RESULTS

3.3.1. Biomass production

The leaf biomass of the *Festuca* line EB15 was significantly affected by salinity, *Epichloë*, *Fusarium*, and the [*Epichloë* × *Fusarium*] interaction (Table 3.1). Leaf biomass decreased with salinity, E+ clones had greater biomass than E− clones regardless of salinity, and inoculation with *Fusarium* increased the leaf biomass in all treatments, but this response was greater in E− than in E+ plants (Figure 3.2A). In addition, root biomass was significantly greater in plants inoculated with *Fusarium* (0.818 ± 0.051 g *vs*. 0.647 ± 0.050 g) regardless of *Epichloë* infection or the salinity treatment (Table 3.1; Figure 3.2B).

In *Festuca* line TH12 only *Periconia* had a significant effect on leaf biomass (Table 3.1). Inoculated plants (0.536 ± 0.049 g) had greater leaf biomass than uninoculated (0.331 ± 0.028 g), regardless of *Epichloë* and salinity (Figure 3.2C). Root biomass was significantly affected by *Periconia*, [*Epichloë* × salt] and [*Epichloë* × *Periconia* × salt] interactions (Table 1). The triple interaction indicated that the positive effect of *Periconia* on root growth was more pronounced in E+ plants at 0 mM NaCl, and in E− plants at 600 mM NaCl (Figure 3.2D).

Table 3.1 - ANOVA results showing the effect of *Epichloë* presence, inoculation and salt treatment on different parameters of *Festuca rubra* subsp. *pruinosa* plants. (In bold significant effects)

		Epichloë (E)	Root endophyte (R)	Salt	R×Salt	E×R	E×Salt	E×R×Salt
Shoot biomass								
line EB15/*F. oxysporum* T48	$F_{7,39}$	6.08	29.5	4.64	1.65	8.06	0.632	0.061
	p	**0.019**	**<0.001**	**0.039**	0.208	**0.008**	0.432	0.808
line TH12/*P. macrospinosa* T131	$F_{7,39}$	0.03	14.3	3.78	2.42	0.092	0.429	1.78
	p	0.862	**<0.001**	0.061	0.129	0.763	0.517	0.191
Root biomass								
line EB15/*F. oxysporum* T48	$F_{7,39}$	1.65	5.51	0.004	0.464	0.079	2.89	0.005
	p	0.207	**0.025**	0.951	0.500	0.780	0.099	0.940
line TH12/*P. macrospinosa* T131	$F_{7,39}$	0.362	20.3	1.67	0.514	0.001	7.44	5.67
	p	0.552	**<0.001**	0.205	0.478	0.980	**0.010**	**0.023**
Proline								
line EB15/*F. oxysporum* T48	$F_{7,23}$	2.04	0.246	187	0.437	0.953	2.44	0.869
	p	0.175	0.628	**<0.001**	0.519	0.346	0.141	0.367
line TH12/*P. macrospinosa* T131	$F_{7,23}$	0.418	1.21	694	0.101	0.705	0.262	0.783
	p	0.529	0.291	**<0.001**	0.756	0.416	0.617	0.392
Na								
line EB15/*F. oxysporum* T48	$F_{7,39}$	1.69	40.1	193	41.1	10.6	2.01	9.16
	p	0.214	**<0.001**	**<0.001**	**<0.001**	**0.006**	0.178	**0.009**
line TH12/*P. macrospinosa* T131	$F_{7,39}$	0.128	9.25	622	8.45	6.9	0.379	6.75
	p	0.726	**0.009**	**<0.001**	**0.012**	**0.021**	0.549	**0.022**
K								
line EB15/*F. oxysporum* T48	$F_{7,39}$	0.429	0.005	25.5	0.096	0.005	0.663	0.663
	p	0.523	0.946	**<0.001**	0.761	0.944	0.429	0.429
line TH12/*P. macrospinosa* T131	$F_{7,39}$	0.357	0.163	24.4	0.682	0	1.31	0.001
	p	0.561	0.693	**<0.001**	0.424	0.997	0.273	0.988
Na:K								
line EB15/*F. oxysporum* T48	$F_{7,39}$	3.74	86.2	414	87.6	25.6	4.06	22.1
	p	0.074	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.064	**<0.001**
line TH12/*P. macrospinosa* T131	$F_{7,39}$	0.355	8.12	319	7.44	4.97	0.146	4.87
	p	0.561	**0.014**	**<0.001**	**0.017**	**0.044**	0.709	**0.046**
Antioxidant capacity								
line EB15/*F. oxysporum* T48	$F_{7,39}$	6.08	29.5	4.64	1.65	8.06	0.63	0.06
	p	**0.019**	**<0.001**	**0.039**	0.208	**0.008**	0.432	0.808
line TH12/*P. macrospinosa* T131	$F_{7,39}$	0.03	14.3	3.78	2.42	0.092	0.429	1.78
	p	0.862	**<0.001**	0.061	0.129	0.763	0.517	0.191
Phenolic compounds								
line EB15/*F. oxysporum* T48	$F_{7,39}$	1.65	5.51	0.004	0.464	0.079	2.89	0.005
	p	0.207	**0.025**	0.951	0.500	0.78	0.099	0.940
line TH12/*P. macrospinosa* T131	$F_{7,39}$	0.362	20.3	1.67	0.514	0.001	7.44	5.67
	p	0.552	**<0.001**	0.205	0.478	0.98	**0.010**	**0.023**

(A)

(C)

(B)

(D)

Figure 3.2 - Leaf and root biomass produced at two different salinity treatments (0 and 600 mM NaCl) in two *Festuca rubra* subsp. *pruinosa* lines, EB15 **(A,B)** and TH12 **(C,D)**, each one composed by clones symbiotic with *Epichloë festucae* (E+) or *Epichloë* free (E−), and inoculated with *Fusarium oxysporum* T48 (orange), *Periconia macrospinosa* T131 (dark red), or uninoculated (green). Where a significant [salinity × *Epichloë* × root endophyte] interaction occurred, different means are indicated by different letters.

3.3.2. Sodium and potassium content

In both FRP lines, a significant effect of salinity, both root endophytes, and the interactions [*Epichloë* × root endophyte], [root endophyte × salinity], and [*Epichloë* × root endophyte × salinity] on Na$^+$ content of leaves were detected (Table 3.1). In both plant lines, Na$^+$ content increased in plants treated with 600 mM NaCl, and in uninoculated plants it was lower in E+ than in E− clones (Figure 3.3A,D). In *Festuca* line EB15 the Na$^+$ content under salinity decreased significantly in plants inoculated with *Fusarium,* but it was affected by the *Epichloë* status. In line TH12 the opposite occurred, at 600 mM NaCl the Na$^+$ content increased in E+ plants inoculated with *Periconia*; however, in E− plants differences between inoculation treatments were not significant (Figure 3.3D).

73

(A)

(D)

(B)

(E)

(C)

(E)
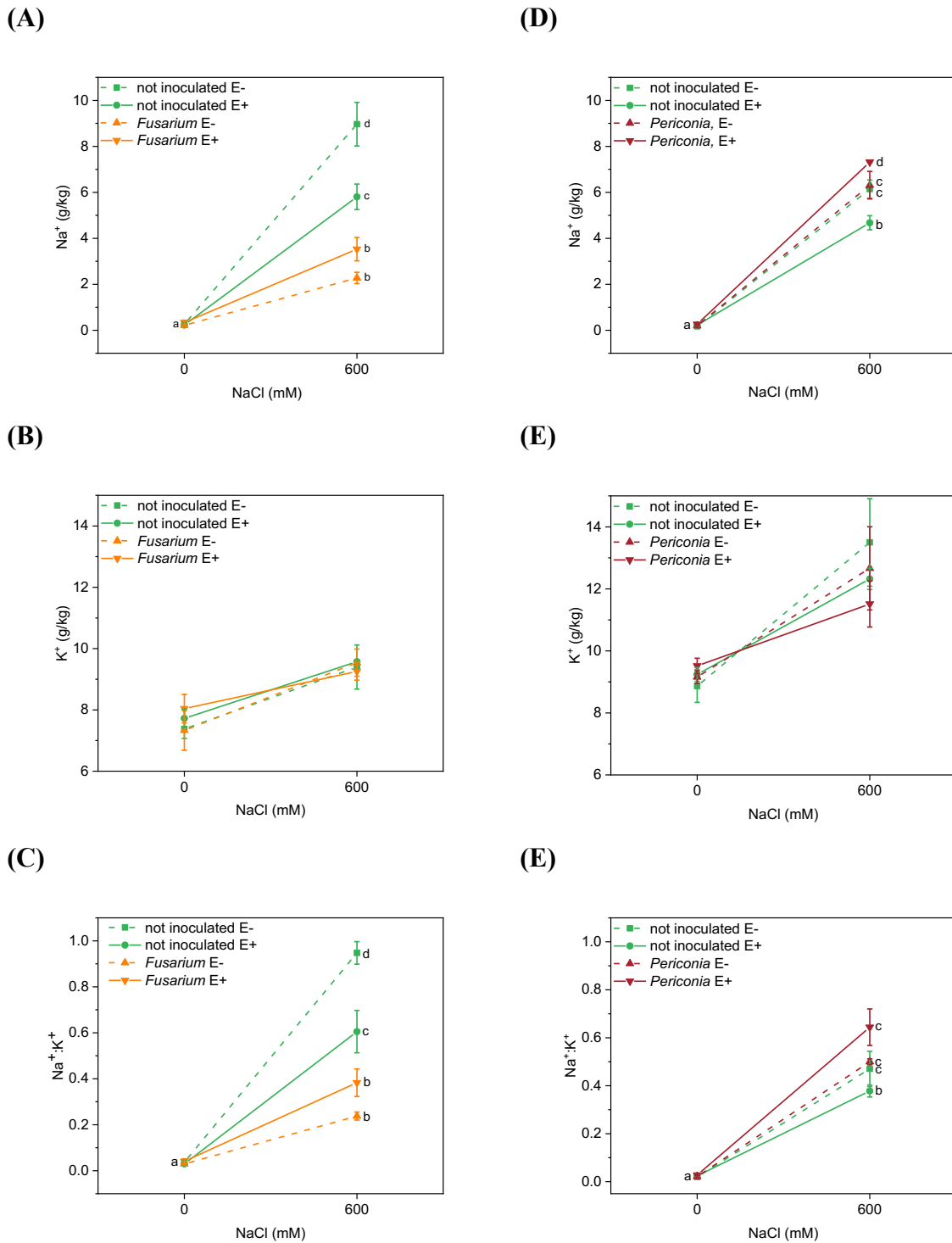
Figure 3.3 - Leaf sodium and potassium content and Na$^+$:K$^+$ ratio in two *Festuca rubra* subsp. *pruinosa* lines, EB15 **(A,B,C)** and TH12 **(D,E,F)**. Each line was composed by clones symbiotic with *Epichloë festucae* (E+) or *Epichloë* free (E−). Plants of each line were inoculated with *Fusarium oxysporum* T48 (orange), *Periconia macrospinosa* T131 (dark red), or uninoculated (green), and subjected to two different salinity treatments (0 and 600 mM NaCl). Where a significant [salinity × *Epichlo*ë × root endophyte] interaction occurred, different means are indicated by different letters.

In both plant lines the K^+ content significantly increased at 600 mM NaCl, although this increase was less pronounced than the one observed for Na^+ (Figure 3.3B,E). Neither the *Epichloë* status nor the root endophytes significantly affected this parameter (Table 3.1).

Mainly driven by the Na^+ response, the Na^+/K^+ ratio significantly increased in leaves of all plants at 600 mM NaCl. In both lines a significant effect of root endophyte inoculation [*Fusarium* and *Periconia*] and the interactions [*Epichloë* × root endophyte], [root endophyte × salinity], and [*Epichloë* × root endophyte × salinity] were detected (Table 3.1). In uninoculated plants of the line EB15 subject to salinity this ratio was significantly lower in E+ than in E− plants, and in plants inoculated with *Fusarium,* Na^+/K^+ further decreased in both E+ and E− (Figure 3.3C). In line TH12 the Na^+/K^+ was also lower in uninoculated E+ plants at 600 mM NaCl, and the inoculation with *Periconia* did not reduce this ratio, as observed with *Fusarium* in the other line (Figure 3.3F).

3.3.3. Proline content

Salinity had a significant effect on the leaf proline content in both FRP lines (Table 3.1, Figure 3.4). Neither *Epichloë* nor the root endophytes affected the content of this osmolyte. The proline content in leaves at 600 mM NaCl was very similar in both lines (EB15: 5.606 ± 0.440 g/kg; TH12: 5.664 ± 0.193 g/kg).

(A)   **(B)**
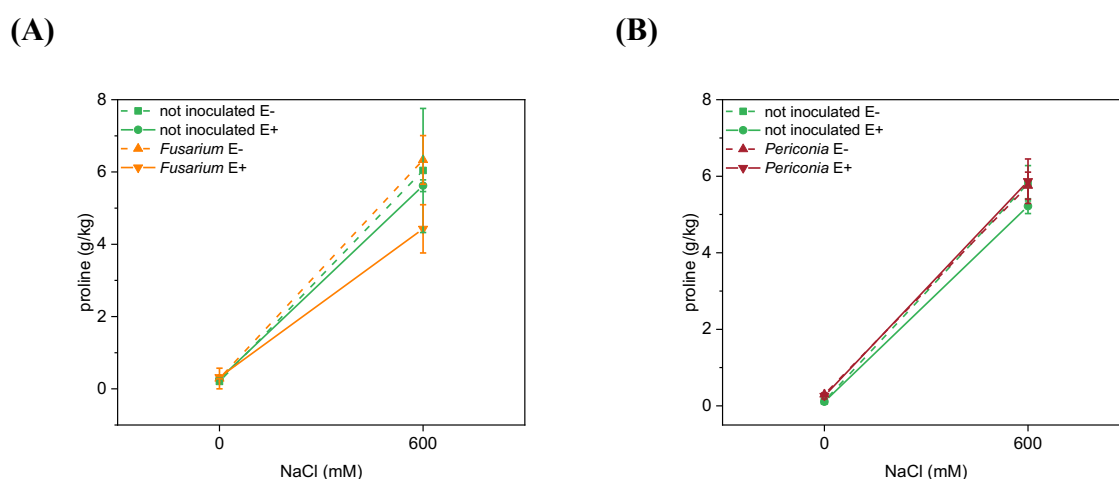
Figure 3.4 - Leaf proline content at two different salinity treatments (0 and 600 mM NaCl) in two *Festuca rubra* subsp. *pruinosa* lines, EB15 **(A)** and TH12 **(B)**, each one composed by clones symbiotic with *Epichloë festucae* (E+) or *Epichloë* free (E−), and inoculated with *Fusarium oxysporum* T48 (orange) or *Periconia macrospinosa* T131 (dark red), or uninoculated (green).

3.3.4. Antioxidant capacity and phenolic compounds content

Both salinity and *Fusarium* factors had a significant effect on the antioxidant capacity and content of total phenolic compounds (TPhC) (Table 3.1, Figure 3.5). Plants inoculated with *Fusarium* had lower antioxidant capacity and TPhC content than uninoculated plants, and both parameters decreased with salinity. *Fusarium* had a significant interaction with *Epichloë*, in inoculated plants the antioxidant capacity was lower in E+ than in E− plants.

The antioxidant capacity of FRP line TH12 was significantly affected by salinity and the [*Epichloë* × salinity] interaction (Table 3.1, Figure 3.5), decreased with salinity in E− plants, but in E+ plants differences between salt treatments were not statistically significant. Inoculation with *Periconia* was the only factor affecting the TPhC content, which significantly decreased in plants infected with the root endophyte, regardless of the *Epichloë* infection and the salt treatment (Table 3.1, Figure 3.5).

(A)

(B)

(C)

(D)
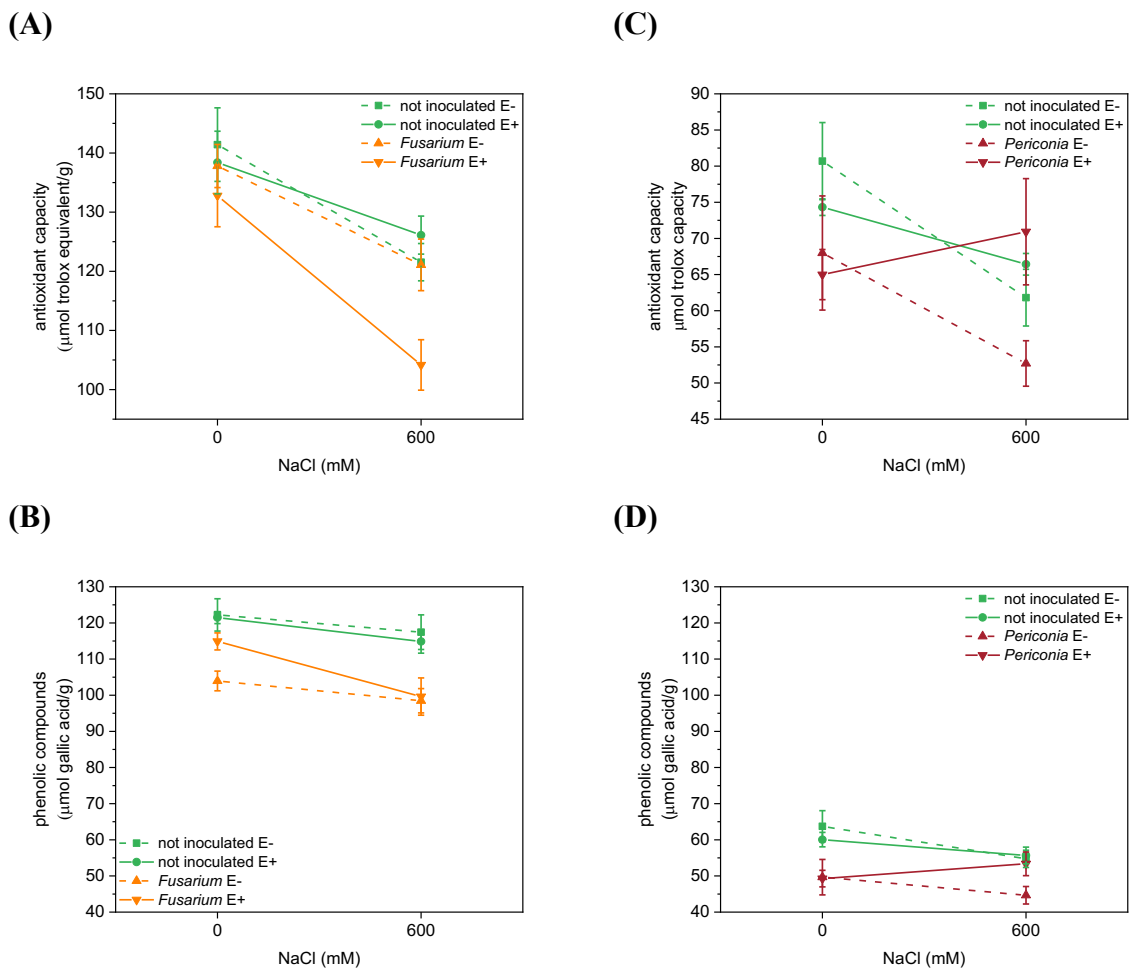
Figure 3.5 - Antioxidant capacity and phenolic compounds content in two *Festuca rubra* subsp. *pruinosa* lines, EB15 **(A,B)** and TH12 **(C,D)**. Each line was composed by clones symbiotic with *Epichloë festucae* (E+) or *Epichloë* free (E−). Plants of each line were inoculated with *Fusarium oxysporum* T48 (orange), *Periconia macrospinosa* T131 (dark red), or uninoculated (green), and subjected to two different salinity treatments (0 and 600 mM NaCl).

3.3.5. Ergovaline content

Epichloë festucae can produce ergovaline in symbiotic FRP plants. Therefore, this alkaloid was analyzed only in E+ plants of each line. Plants inoculated with *Periconia* or uninoculated had ergovaline contents below the limit of quantification (0.02 µg/g), suggesting that this FRP −*Epichloë* combination does not produce ergovaline. In plants of line EB15 there was a significant positive effect of salinity (F= 6.02¸ *p*= 0.044) on ergovaline content, but the effect of *Fusarium* was not statistically significant (Figure 3.6).
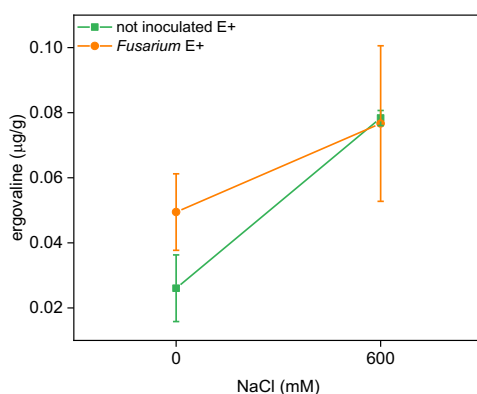
Figure 3.6 - Ergovaline concentration in leaves of *Festuca rubra* subsp. *pruinosa* line EB15 symbiotic with *Epichloë festucae* (E+), inoculated with *Fusarium oxysporum* T48 (orange) or uninoculated (green), and subjected to two different salinity treatments (0 and 600 mM NaCl).

3.3.6. Root microscopy

The presence of fungal structures in inter and intracellular spaces indicated the presence of the root endophytes and successful plant inoculation (Figure 3.7). In roots of plants inoculated with *Periconia*, melanized septate hyphae and microsclerotia were observed (Figure 3.7A,B). These structures are typical of dark septate endophytes. In plants inoculated with *Fusarium* T48, hyphae were observed in the root cortex (Figure 3.7C,D).
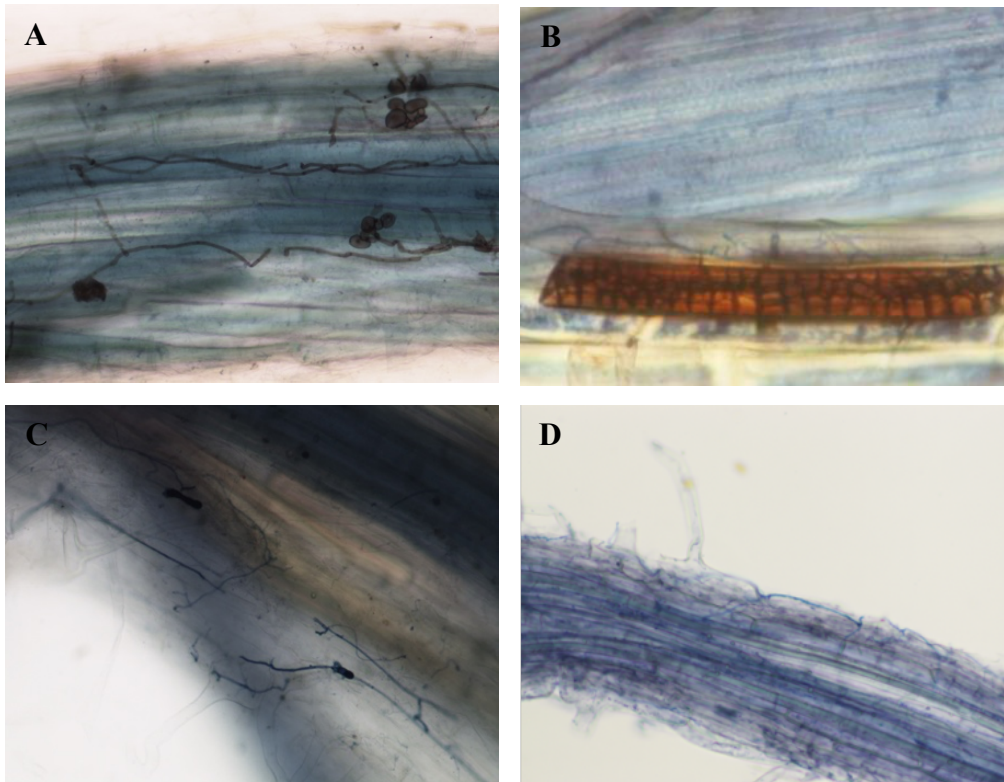
Figure 3.7 - Fungal structures observed by light microscopy in roots of plants inoculated with *Periconia macrospinosa* **(A,B)** and *Fusarium oxysporum* **(C,D)**. In roots inoculated with *Periconia* melanized septated hyphae and spores **(A)** and microsclerotia **(B)** are visible. In *Fusarium*-inoculated plants hyphae are visible in the root cortex.

3.3.7. *In vitro* interaction between *Epichloë festucae* and root endophytes

Dual cultures of *E. festucae* strains isolated from the FRP lines and the root endophytes *P. macrospinosa* and *F. oxysporum* were established. The results showed that *E. festucae* had an inhibitory effect on the mycelial growth of *P. macrospinosa* (Figure 3.8A). However, this pattern was not observed with *F. oxysporum*, whose mycelium grew over the *E. festucae* colony (Figure 3.8B).

In dual cultures with *Periconia* and *Fusarium* the radial growth of *E. festucae* was stimulated*,* increasing by 42% and 48% respectively, relative to the dual cultures of *E. festucae* alone (Figure 3.8A,D).
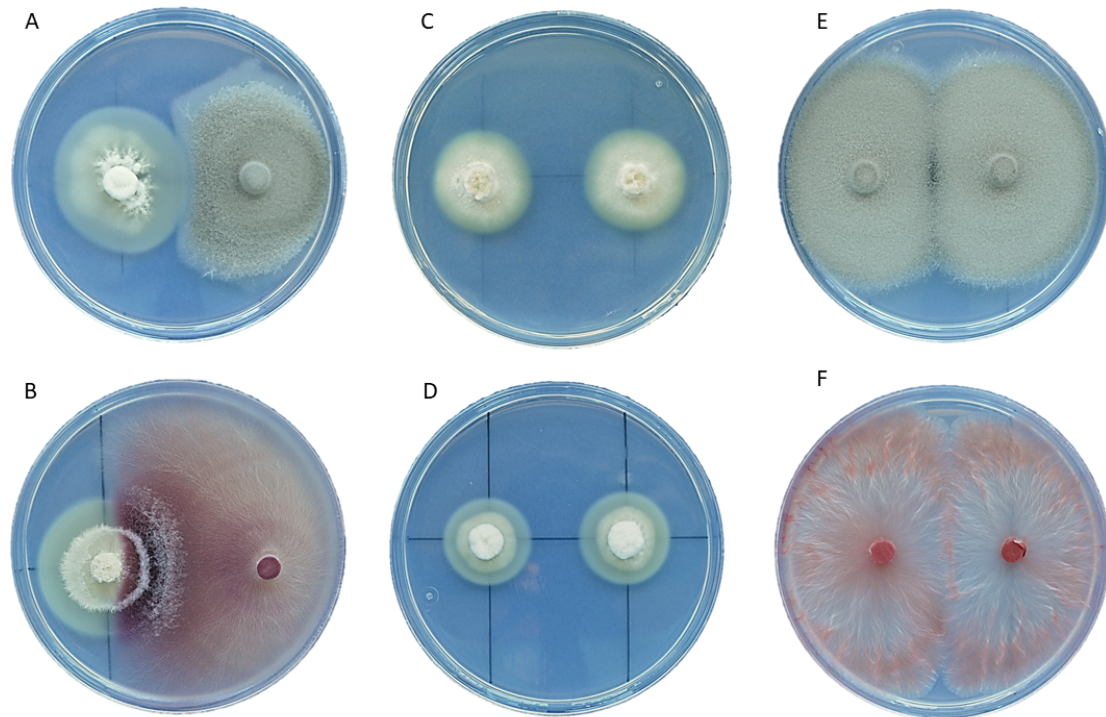
Figure 3.8. Dual culture interaction between *Epichloë festucae* and the root endophytes *Periconia macrospinosa* T131 **(A)**, and *Fusarium oxysporum* T131 **(B)**. Dual culture control of *Epichloë* EB15 **(C)**, *Epichloë* TH12 **(D)**, *Periconia macrospinosa* T131 **(E)**, and *Fusarium oxysporum* T131 **(F)**. All cultures are two weeks old.

3.4. DISCUSSION

3.4.1. Plant characteristics related to salinity tolerance

The halophytic character of *Festuca rubra* subsp. *pruinosa* (FRP) was clearly observed in its response to salinity. The biomass reduction caused by exposure to high salinity was low, being statistically significant only for the leaf biomass of line EB15, while the root growth was not significantly affected in any case. Regarding this, shoot growth is reported as more sensitive to salinity than root growth in *Festuca rubra*, as well as in other plant species (Baumeister and Merten, 1981; Munns and Tester, 2008).

The response of ion and solute accumulation to salinity stress gives an insight into some mechanisms of adaptation of FRP to its natural environment in sea cliffs. As previously reported for *F. rubra* subsp. *litoralis* (Rozema et al., 1978), exposure to salinity caused a substantial increment in the shoot content of Na^+ and proline in FRP. This type of response occurs in plant species having tissue tolerance to Na^+. In contrast to the alternative mechanism of Na^+ exclusion from leaf blades, plants having tissue tolerance accumulate Na^+ in their tissues

by sequestering it into cell vacuoles. When this occurs, an osmotic adjustment of the cytoplasm is needed to maintain cell turgor, and this is achieved by the synthesis and accumulation of proline or other compatible solutes, as well as K^+ (Flowers and Colmer, 2008; Munns and Tester, 2008; Zhao et al., 2020). This balance with proline, a common osmolyte among halophytic grasses (Slama et al., 2015), occurred in FRP regardless of the presence of leaf or root symbionts. A significant increase in K^+ leaf concentration also occurred in response to salinity, independently of the symbiotic status of the plants. Different responses in terms of K^+ content have been observed in different halophytes, some maintain the K^+ concentration regardless of salinity, while others, including some maritime halophytes like FRP, show increased K^+ content under salinity as a stress tolerance strategy (Flowers and Colmer, 2008; Assaha et al., 2017).

Both FRP lines differed in salinity tolerance. Salinity had a significant negative effect on the leaf biomass of line EB15, but not on TH12. At the same time, the Na^+ content of line TH12 was lower than that of EB15. A similar inverse relation between salinity tolerance and Na^+ content was reported to occur among subspecies of *F. rubra*, with the most tolerant subspecies having less Na^+ content than the least tolerant inland subspecies (Rozema et al., 1978). Tolerance to salinity is an inheritable character in *Festuca rubra* (Humphreys, 1982), and the mechanisms related to the management of cellular Na^+ are very likely to be related to it.

3.4.2. *Epichloë* effects on plants subject to salinity

Because of salinity, scarcity of soil and nutrients, and frequent wind exposure, the FRP habitat in sea cliffs could be considered suboptimal for plant growth. Considering that the incidence of *Epichloë festucae* is relatively high in FRP populations, about 66% (Chapter 2, Pereira et al., 2019), it could be expected that in such demanding habitat the benefits from this symbiosis must be greater than its costs for the plant in order to support such incidence rates in a vertically inherited symbiont.

While some works report a clearly beneficial effect of *Epichloë* endophytes on several parameters related to salt tolerance in grasses (Song et al., 2015; Wang et al., 2020), others indicate that beneficial effects might be dependent on the grass-endophyte genotypes (Sabzalian and Mirlohi, 2010). In terms of leaf or root biomass, our results do not support an obvious or unambiguous role of *Epichloë* itself in FRP salt tolerance, confirming the results of a previous experiment (Zabalgogeazcoa et al., 2006b). However, the accumulation of Na^+ was significantly lower in leaves of E+ than in E− plants of both plant lines. Such reduction in Na^+

accumulation under salinity might be advantageous for salinity tolerance and habitat adaptation. In fact, among subspecies of *F. rubra* the tolerance to salinity appears to be inversely correlated to Na^+ accumulation (Rozema et al., 1978). A similar effect of Na^+ reduction in plants symbiotic with *Epichloë* was also reported in other grass species (Sabzalian and Mirlohi, 2010; Song et al., 2015). Nevertheless, other important fitness parameters such as seed production or germination have not been examined in relation to the *Epichloë* status of plants, and such information could help to understand the high prevalence of this symbiosis in sea cliff populations.

Plant protection against herbivores mediated by fungal alkaloids like ergovaline is often cited as a main driver of *Epichloë*-grass symbioses (Schardl et al., 2013). However, macroherbivores are absent from sea cliffs, and herbivore protection mediated by fungal alkaloids is not a satisfactory hypothesis to understand why *Epichloë* infection rates are high in sea cliff populations. It is worth noting that the incidence of *Epichloë* in *F. rubra* populations from sea cliffs is very similar to that observed in inland populations from semiarid grasslands (Zabalgogeazcoa et al., 1999; Chapter 2, Pereira et al., 2019). Perhaps other unknown factors than herbivory are as important for the favorable selection of these symbiotic associations.

Only plants of the line EB15 contained the fungal alkaloid ergovaline. Phenotypic variation in ergovaline content occurs among FRP plants, in a previous survey 21% of the plants analyzed did not contain this alkaloid (Vázquez de Aldana et al., 2007). Lack of ergovaline in symbiotic plants could be due to the absence of functional genes in the biosynthetic pathway, plant-fungal compatibility, environmental effects, or both (Schardl et al., 2013; Vázquez de Aldana et al., 2020b). The ergovaline content increased greatly in EB15 plants subject to salinity. Increased content of diverse alkaloids in response to salinity has been reported in several plant species (Wang et al., 2010; Li et al., 2020), including some ergot alkaloids produced by *Epichloë* in the grass *Achnatherum inebrians* (Zhang et al., 2011). Whether *Epichloë* alkaloids could have a function other than defensive in plants is unknown. The antioxidant capacity of FRP plants under salinity stress did not differ in response to *Epichloë* symbiosis itself (in absence of root endophytes). This result contrasts with those of Chen et al. (2018), who observed an increment in total antioxidant activity in E+ plants of *Hordeum brevisubulatum* subject to salinity. The fact that the functional intensity of the antioxidant machinery is time dependent, and several enzymes peak soon after stress exposure, but later return to a basal level (Baltruschat et al., 2008), might explain this difference. Alternatively, FRP is a halophytic marine plant species, and has an efficient endogenous

machinery to cope with salinity, where accumulation of antioxidants might not be responsible for the salt stress adaptation.

3.4.3. Root endophyte effect on plants subject to salinity

The most remarkable results from this study are the observation that both *F. oxysporum* and *P. macrospinosa,* prevalent root endophytes in natural populations of FRP, have a significant beneficial effect on plant growth, regardless of the salinity treatment. Thus, these two fungal components of the FRP microbiome could favor plant hosts in their native habitat. *Fusarium oxysporum* is the most abundant fungal species detected in the root endosphere of FRP, with a prevalence of 57% in natural populations, is a likely component of the core microbiome of this grass (Chapter 2, Pereira et al., 2019). Asymptomatic root infections like the ones we observed in FRP plants are common for many *Fusarium* species (Bacon and Yates, 2006). *Fusarium oxysporum* is best known for its pathogenic *formae speciales*, but also contains numerous strains having an endophytic lifestyle, like those present in FRP roots (Demers et al., 2015; Edel-Hermann and Lecomte, 2019). Endophytic, non-pathogenic, *F. oxysporum* strains may act as biocontrol agents against several root pathogens, including pathogenic strains of their own species (Constantin et al., 2020; de Lamo and Takken, 2020), and in some circumstances might promote plant growth in the absence of disease (Bitas et al., 2015).

Root endophytes like *Serendipita indica* or *Fusarium culmorum* have been reported to increase host plant tolerance to salinity (Waller et al., 2005; Rodriguez et al., 2008). The latter is a dominant endophyte in several organs of the beach grass *Leymus mollis*, and probably it is an important microbiome component for the adaptation of the plants to their maritime habitat (Rodriguez et al., 2008). In view of our results, a similar function could be expected from *F. oxysporum* endophytes in FRP plants.

A parameter having a remarkable response in FRP plants inoculated with *F. oxysporum* was the Na^+ content of leaves, which showed a pronounced decrease when compared to uninoculated plants. This response did not occur in plants inoculated with *P. macrospinosa*. As explained before, the results from our experiment suggest that in the absence of *F. oxysporum*, FRP plants cope with salinity by means of a tissue tolerance mechanism consisting of the vacuolar sequestration of Na^+. However, plants also have other mechanisms to cope with salinity, like the exclusion of Na^+ from entering the plants, a mechanism centered in plant roots (Munns and Tester, 2008; Zhao et al., 2020). Thus, the association with *F. oxysporum* could

be limiting Na$^+$ uptake from plant roots by means of modulating or complementing the plant Na$^+$ exclusion machinery. A similar situation of symbiont mediated Na$^+$ exclusion has been reported in *Arabidopsis* inoculated with *Serendipita indica* (Abdelaziz et al., 2019; Lanza et al., 2019).

In addition, the growth promotion observed in the absence of salt in plants inoculated with *F. oxysporum* might be related to nutrient acquisition, an important issue in a habitat where soil is scarce or absent. Improved nutrient acquisition mediated by symbiotic fungi could occur in several ways, for instance, root endophytes could help to recycle dead plant material (Upson et al., 2009; Vázquez de Aldana et al., 2013), produce plant hormones that stimulate root growth (Sirrenberg et al., 2007), or alter the chemistry or microbiota of the rhizosphere (Alegría Terrazas et al. 2016). Thus, the increased shoot biomass observed in inoculated plants could be due to the greater root size caused by hormonal stimulation, increased nutrient availability, or both together mediated by *F. oxysporum*.

Periconia macrospinosa is a DSE with a wide host range, which has been described in roots of numerous grasses and other plants (Mandyam et al., 2012). In natural populations of FRP the incidence of this taxon was about 16%, being one of its most abundant root endophytes (Chapter 2, Pereira et al., 2019). The search for the ecological function of DSE symbioses has been elusive, although some studies report increased biomass and nutrient (N, P) content, as well as salt tolerance in host plants (Mandyam and Jumpponen, 2005; Newsham, 2011; Gonzalez Mateu et al., 2020). In the present study, *P. macrospinosa* caused a significant increase in leaf and root biomass of its original host plant, FRP, in the presence as well as in the absence of salinity stress. *Periconia macrospinosa* is known to produce a wide range of extracellular enzymes able to metabolize numerous substrates, organic as well as inorganic (Mandyam et al., 2010; Knapp and Kovács, 2016). This fungus, which is thought to have a life cycle as a latent saprobe, could be involved in nutrient cycling and mobilization (Yakti et al., 2018). In the habitat of FRP plants, where soil is often nonexistent, nutrient cycling from dead plants or other organic remains could be very important for habitat adaptation. In contrast with *F. oxysporum*, this fungus did not reduce the Na$^+$ content of the plants, therefore, as a symbiont its contribution might not be related to salinity tolerance.

3.4.4. Interactions among holobiont components

Festuca rubra is a mainly outcrossing species that can also reproduce asexually by means of vegetative tillers produced from rhizomes (Harberd, 1961). Thus, populations of *F.*

rubra could be conceived as groups of genotypically distinct individuals, some of which could be more or less represented by means of clonal expansion. As we found in this study, distinct plant genotypes can differ in their tolerance to salinity, an important trait for adaptation to sea cliffs. *Epichloë festucae* endophytes interact with plant genotypes in *Festuca* populations. As observed, the response to salinity of the plant individuals can be modified by their interaction with *Epichloë* endophytes, which reduce their Na^+ accumulation. Thus, the adaptation to salinity of both plant genotypes might be augmented by symbiosis with *Epichloë*. Further interactions seemed to occur with root endophytes. For instance, in terms of root growth and Na^+ accumulation, *P. macrospinosa* was positive for E−, but not for E+ plants, and the trend seemed to be opposite in the case of *F. oxysporum*. In addition, although *Epichloë* and root endophytes occupy different plant compartments, the dual culture experiments suggested that *E. festucae* cultures respond to the presence of both root endophytes. In these experiments, the growth of *Epichloë* was stimulated by the presence of the root endophytes, and *Periconia* was inhibited by *Epichloë*, but *Fusarium* was not. Taking into account that more than 100 species of culturable fungi have been identified in FRP roots (Chapter 2, Pereira et al., 2019), multiple interactions among microbiome components are likely to have important effects on distinct plant genotypes. Such a complex landscape of interactions affecting holobiont performance might help to understand why FRP populations having a 100% incidence of *E. festucae* are rare in marine and inland ecosystems. Even if *E. festucae* is beneficial for some symbiotic plants, other holobiont configurations might compensate for its absence.

3.5. CONCLUSIONS

This study sheds light on how a plant supported by its microbiome can adapt to an inhospitable habitat in sea cliffs. To cope with salinity *Festuca rubra* subsp. *pruinosa* seems to rely on a tissue tolerance mechanism that allows its cells to accumulate Na^+, possibly in vacuoles, and an osmotic counterbalance occurs in the cytoplasm by means of proline and K^+. In addition to these intrinsic plant mechanisms, *Epichloë festucae*, *Fusarium oxysporum* and *Periconia macrospinosa*, three fungal endophytes highly prevalent in natural populations of *F. rubra*, also contributed to improve plant performance under salinity. *Epichloë* caused a Na^+ reduction in leaves under salinity, which might be associated with salinity tolerance and plant survival in the long term. *Fusarium oxysporum*, the most abundant root endophyte from *F. rubra* appears to contribute two different adaptive functions to symbiotic plants: first,

promotion of the growth of leaves and roots in the presence as well as in the absence of salinity; and second, it caused a decrease in leaf Na^+ content under salinity, a function which as above suggested for *Epichloë,* could improve plant adaptation to salinity. *Periconia macrospinosa* promoted the growth of leaves and roots of *F. rubra* plants regardless of the salinity treatment. Although the mechanisms mediating growth promotion or salinity tolerance are unknown, each of these three components of the *F. rubra* core mycobiome contributed different functions, which are beneficial for plant adaptation to its habitat in sea cliffs, supporting our initial hypothesis.

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Chapter 4

Differentially expressed genes and metabolomic changes induced by salt and *Epichloë festucae* in *Festuca rubra* subsp. *pruinosa*

Abstract

Plants of *Festuca rubra* subsp. *pruinosa* inhabit sea cliffs and maintain a symbiotic association with the fungal endophyte *Epichloë* festucae. The plant transcriptome response and the metabolomic changes in response to salinity and the symbiosis with *Epichloë* were investigated. A line of *Festuca rubra* consisting of *Epichloë* infected and *Epichloë* free clones was subjected to salinity, and Illumina high-throughput RNA sequencing of leaves from each treatment was performed to identify differentially expressed genes (DEGs). A set of 2304 plant DEGs was identified and ordered into four expression profile groups. The results showed that salinity had a major effect on gene expression, while the presence of *Epichloë* appeared to have a weak but clear modulating effect on salt-related gene expression. The transcriptome analysis supports that *Festuca rubra* plants adapt to salinity through osmotic adjustment, resulting in increased expression genes for proline biosynthesis and ion transport. Photosynthesis was affected by salinity, as observed by the downregulation of genes encoding for light-harvesting proteins, which can be understood as a strategy to prevent photooxidative damage. An untargeted LC−MS metabolomic analysis showed that the most abundant compounds had relative mass defects consistent with terpenoid compounds. Two compounds were potentialized by the presence of *Epichloë*, regardless of the salinity regime. In conclusion, the present data presented suggest that, firstly, salinity was the major factor affecting gene expression and, secondly, as observed previously, tolerance to salinity was an inherent trait of *Festuca rubra*. Although the effect of *Epichloë* on gene expression was weak, a clear effect was observed on the abundance of some metabolites.

4.1. INTRODUCTION

Plants are symbiotic with fungi and other microorganisms, which may influence their performance (Vandenkoornhuyse et al., 2015; Trivedi et al., 2020). Fungi are very diversified, at structural and functional levels, and adopt different trophic strategies, being able to colonize different plant tissues. Fungal endophytes colonize the internal tissues of their host plants without causing visible symptoms (Wilson, 1995; Stone et al., 2000). Species of the genus *Epichloë* belong to the Clavicipitaceae family and colonize systemically the intercellular space of aboveground plant tissues of grasses (Poaceae) (Clay and Schardl, 2002; Schardl et al., 2004).

Festuca rubra subsp. *pruinosa* (FRP) is a perennial grass common in sea cliffs that maintains a symbiotic association with *Epichloë festucae*, (Zabalgogeazcoa et al., 2006; Chapter 2, Pereira et al., 2019). In Chapter 2 it was determined that the incidence of *E. festucae* is relatively high in FRP populations, about 66%, and therefore it should be expected that in a demanding habitat such as sea cliffs, the benefits of this symbiosis are greater than its costs for the plant to support this symbiont. In Chapter 3 it was shown that FRP is well adapted to salinity conditions, and the symbiotic association with *E. festucae* can cause a reduction in Na^+ content of leaves under salinity. An important characteristic of the symbiotic interaction of FRP with *E. festucae* is the production of secondary metabolites, mainly alkaloids (Vázquez de Aldana et al., 2007; 2020). In Chapter 3, it was shown that in one FRP line the ergovaline alkaloid content increased under salinity. Despite the results obtained in Chapter 3, it is still unclear whether *E. festucae* can provide more benefits to FRP plants for adaptation to salinity.

The compatibility and specificity between *Epichloë* and its host are hereditary and complex (Gundel et al., 2010). The sequencing of mRNA using next-generation sequencing technologies (RNA-seq) has the potential to reveal the unique complexity of transcriptional responses to biotic and/or abiotic changes in plants (Garg and Jain, 2013). Previous studies have demonstrated the presence of differentially expressed genes (DEGs) in *Lolium* and *Festuca* species in response to *Epichloë* (Johnson et al., 2013; Ambrose et al., 2012; Dinkins et al., 2012; 2017; Nagabhyru et al., 2019), as well as to salinity (Diédhiou et al., 2009; Amombo et al., 2018), and the effect of *Epichloë* on adaptation to drought (Dinkins et al., 2019). Additionally, metabolomics is also an important tool to gain a comprehensive perspective of how metabolic networks are regulated and respond to changes caused by abiotic stress or by symbiotic relationships in plants. For example, metabolomic techniques revealed

metabolic changes in the plant responses to salinity (Kumari et al., 2015; Carrera et al., 2021). Furthermore, it can also provide an insight into changes in primary and secondary metabolism in the grass-*Epichloë* metabolome (Cao et al., 2008; Green et al., 2020).

Thus, the objectives of this work were double: (1) to monitor gene expression in clones of *Festuca rubra* subsp. *pruinosa* infected with *E. festucae* (E+) and without *E. festucae* (E−) under different salinity treatments, tap-water (0 mM NaCl) and salt-water (600 mM NaCl) in order to identify plant genes that were responsive to the endophyte and salinity and (2) to analyze the metabolome of the same FRP clones and so as to identify metabolite changes caused by salinity and *E. festucae* infection.

4.2. MATERIALS AND METHODS

4.2.1. Plant and fungal material

Plants of *Festuca rubra* subsp. *pruinosa* were originally collected from sea cliffs on the northern coast of Galicia, Spain (Chapter 2, Pereira et al., 2019). A FRP line (CD8), consisting of clonal plants differing from being infected by *Epichloë festucae* (E+) or *Epichloë*-free (E−), was developed as explained in Chapter 3.

4.2.2. Experimental design

To study differential gene expression between E+ and E− clones of FRP at two salinity levels, an experiment was conducted using the isogenic line CD8. A bioassay was designed with four treatments, each one with three plant replicates: E− plants at 0 mM NaCl (S0_ E−), E+ plants at 0 mM NaCl (S0_E+), E− plants at 600 mM NaCl (S1_ E−) and E+ plants at 600 mM NaCl (S1_E+). Plants subjected to salinity treatment were watered with 300 mM NaCl on the first day to avoid salt shock and then with 600 mM NaCl (corresponding to the salt concentration of sea water) on the following days. Then, leaves from each plant were harvested one week after the first salinity treatment. Leaves were cut to 5 mm long pieces and immediately frozen in liquid nitrogen and stored at −80 ºC for subsequent analyses.

4.2.3. RNA sequencing

The plant samples from each treatment, a total of 12 FRP plants, were sent to the Genomic Sciences Laboratory of North Carolina State University for RNA extraction and sequencing. RNASeq libraries were sequenced on the Illumina NextSeq (500) platform in 150 bp paired-end mode. An initial quality check of the raw sequence data was executed using FastQC v.0.11.3 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Adapter sequences and low-quality terminal nucleotides were removed by Trimmomatic (v0.33) (Bolger et al., 2014). Read pairs longer than 60 nt were kept for further processing.

The *de novo* assembler Trinity (v2.3.2) (Grabherr et al., 2011) was used to assemble separately trimmed short reads. Trimmed short reads for the four different treatments were merged to create a reference transcript assembly.

4.2.3. Functional annotation of protein coding transcripts

Protein-coding transcript sequences were predicted on transcript assembly contigs using GeneMark S-T (Tang et al., 2015). Predicted protein sequences were subjected to BLASTP searches against reference protein sequences on a local server. An E-value cut-off of 1e-3 was applied, and top hit sequences were collected for further comparative analyses. For target reference sequences a reduced redundancy plant protein sequence database (all UniProtKB/TrEMBL Viridiplantae protein sequences clustered at 75% similarity level, resulted in 4,098,635 sequences) plus 5, 53,231 sequences from UniProt/SwissProt were used. Taxonomy information was assigned to UniProt top hits using custom scripts. Protein sequences were further analyzed using the InterPro databases for their protein family relationships, signal peptides and transmembrane domains, and Gene Ontology (GO) terms via local InterPro searches (InterProScan-5.16-55.0, Jones et al., 2014). GO-term information was collected from the InterProScan outputs using custom scripts.

GO term enrichment analysis was carried out with the Python library GOATOOLS (Klopfenstein et al., 2018). GO-term categories that were statistically overrepresented in DEGs when compared to the total set of expressed genes were identified by multiple comparisons at a false discovery rate (FDR) of 0.05 using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995).

4.2.4. Expression quantification and differential expression analysis

Trimmed reads were mapped on the chromosome-scale genome assembly Lolium_2.6.1 of *Lolium perenne* (Nagy et al., in preparation) using HISAT2 (Kim et al., 2015) for producing spliced RNA-seq alignments. PCR duplicates were removed from the resulting bam format alignments using the bammark duplicates tool of the biobambam2 package (https://gitlab.com/german.tischler/biobambam2). Short read alignments were processed by StringTie (v1.34b, Pertea et al., 2015) to obtain transcript counts and abundances. Gene based read count information was extracted from the transcript abundance files by the prepDE.py script of the StringTie package. Differential gene expression analysis was made with EdgeR (Gentleman et al., 2004).

To identify DEGs the likelihood ratio test (LRT) method was used, as implemented in the edgeR package. Treatment combinations (like S0_E−, S0_E+, S1_E− and S1_E+) were contrasted as equal−level treatments (Combination design). Normalized log2 transformed gene-level readcount differences were considered to report DEGs with at least twofold changes with a False Discovery Rate (FDR) of 0.5.

4.2.5. Untargeted metabolomic profiling

Methanolic extracts from freeze-dried FRP leaves were prepared with four plant replicates of each treatment. Approximately, 40 mg of freeze-dried leaves were extracted overnight in 500 µl of 80% HPLC-grade methanol and 20% water at a 1:20 w/v ratio, on a rotary shaker at room temperature. Samples were centrifuged at 15,000 g and the supernatant was transferred to amber vials. Ergotamine was used as a reference for peak alignment. Chromatographic separation of metabolites was performed using a 10 min gradient using a 2.1 × 100 mm (1.7 µm) BEH C18 Ultra-Performance Liquid Chromatography (UPLC) column on a Waters Acquity ultra-high pressure LC system (Waters Corp., Milford, MA, USA). A linear solvent gradient of acidified water (0.1% acetic acid) (phase A) and acetonitrile (phase B) was used as follows: 0 min, 1% B; 1 min, 1% B; 8 min, 99% B; 9 min, 99% B; 9.01 min, 1% B; 10 min, 1% B). The column temperature was set at 40 °C and the flow rate was 0.4 ml min^{-1}.

Mass spectra were acquired using either positive or negative-ion mode electrospray ionization on a Xevo G2-XS quadrupole time-of-flight mass spectrometer (ESI-QToF-MS) (Waters Corp.), over m/z 50–1500 using continuum data acquisition with a mass resolution (M/ΔM, full-width half maximum) of ~ 22,000. MSE spectra were acquired in low- and high-energy collision conditions using a collision energy ramp from 20 to 80 V in the latter. Other

parameters include capillary voltage of 3.0 kV in positive ion mode and 2.0 kV in negative ion mode, desolvation temperature of 350 °C, source temperature of 100 °C, cone gas (N_2) at 25 l h^{-1}, and desolvation gas (N_2) at 600 l h^{-1}. Continuous infusion of the lock mass compound leucine encephalin was performed to allow correction for mass drift. The fragmentation patterns of ions of interest were further investigated using the same equipment in MS/MS analyses, using the same chromatography method as described above. MassLynx RAW files were imported into the Progenesis QI software (Nonlinear Dynamics, Newcastle upon Tyne, United Kingdom) for preprocessing, peak alignment and picking and abundance normalization. Relative mass defect (RMD) values were calculated as in Ekanayaka et al. (2015).

4.3. RESULTS

4.3.1. *Festuca rubra* subsp. *pruinosa* transcriptome assembly

De novo assembly using a total of 192 million paired-end Illumina reads resulted in 1,380,279 transcript assembly contigs. BLASTP hits were obtained for 232,718 transcript assembly contigs. Most of the transcripts (221,784 sequences) belonged to the kingdom Viridiplantae and the remaining ones belonged to sequences of non-plant origin (Table 4.1).

Table 4.1 - Classification of *Festuca rubra* subsp. *pruinosa* assembled transcripts at the kingdom level classification. The numbers represent number of transcripts associated to each kingdom level.

Kingdom	No. of transcripts
Viridiplantae	221,784
Fungi	7,494
Metazoa	3,013
Bacteria	269
Unknown	123
Viruses	18
Archea	17
Total	232,718

4.3.2. Differential gene expression analysis

The *de novo* assembly contained too many contigs, and the assembly was fragmented. Moreover, it was difficult to separate the plant and fungal transcript. Therefore, the *Lolium*

perenne genome (Nagy et al. unpublished) was used as reference because no high-quality genome is available for *Festuca*.

To obtain an overview of the relationship among the samples from each treatment, a principal component analysis (PCA) was conducted with transcripts of plant origin. The results of the PCA revealed differences in transcript expression patterns among the treatments, showing that, firstly, samples from the same treatment clustered together (Figure 4.1), except for one sample belonging to the S1_E+ treatment that was disclosed from further analyses, and, secondly, there was a close similarity between S1_E− and S1_E+. After the S1_E+ sample was eliminated, the first two principal components explained 84% of the total variation.
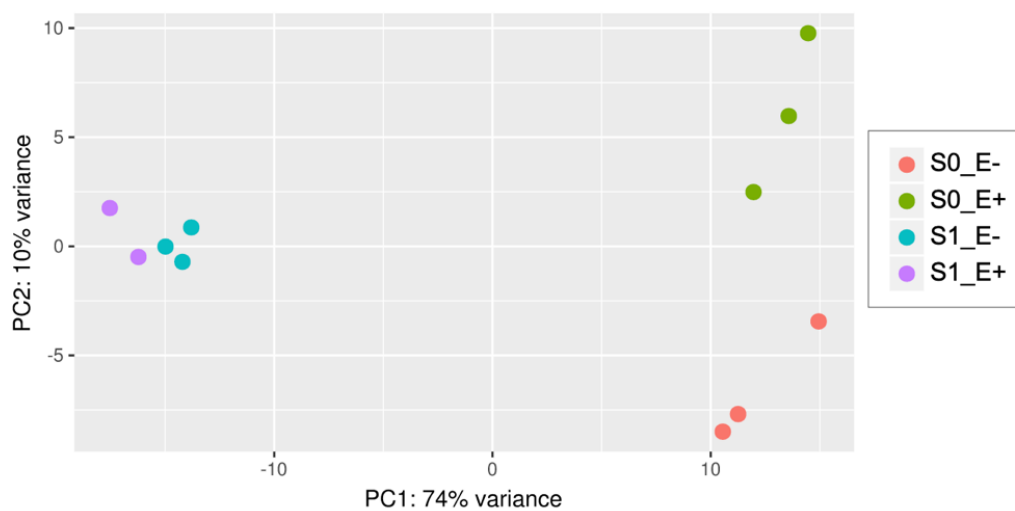
Figure 4.1 – Principal component analysis (PCA) of the plant transcriptome data. [S0_E−]: 0 mM NaCl and without *Epichloë*; [S0_E+]: 0 mM NaCl and infected with *Epichloë*; [S1_E−]: 600 mM NaCl and without *Epichloë*; [S1_E+]: 600 mM NaCl and infected with *Epichloë*. A sample belonging to the S1_E+ treatment was disclosed because it was an outlier.

By comparing the gene expression levels between treatments, the DEGs were determined. A set of 2304 differentially expressed genes was identified, and a hierarchical clustering ordered these DEGs into four expression profile groups (S1 to S4), as shown in the gene expression heatmap (Figure 4.2). Of these expression profile groups, Subcluster 1 (S1= 206 genes) and Subcluster 2 (S2= 503 genes) contained genes up-regulated by salt, while Subcluster 3 (S3= 1457 genes) and Subcluster 4 (S4= 138 genes) contained genes down-regulated by salt. Interestingly, these results showed that salinity had a major effect on gene expression, while the presence of *Epichloë* endophyte seemed to slightly modulate the salt-related gene expression (Figures 4.2 and 4.3).
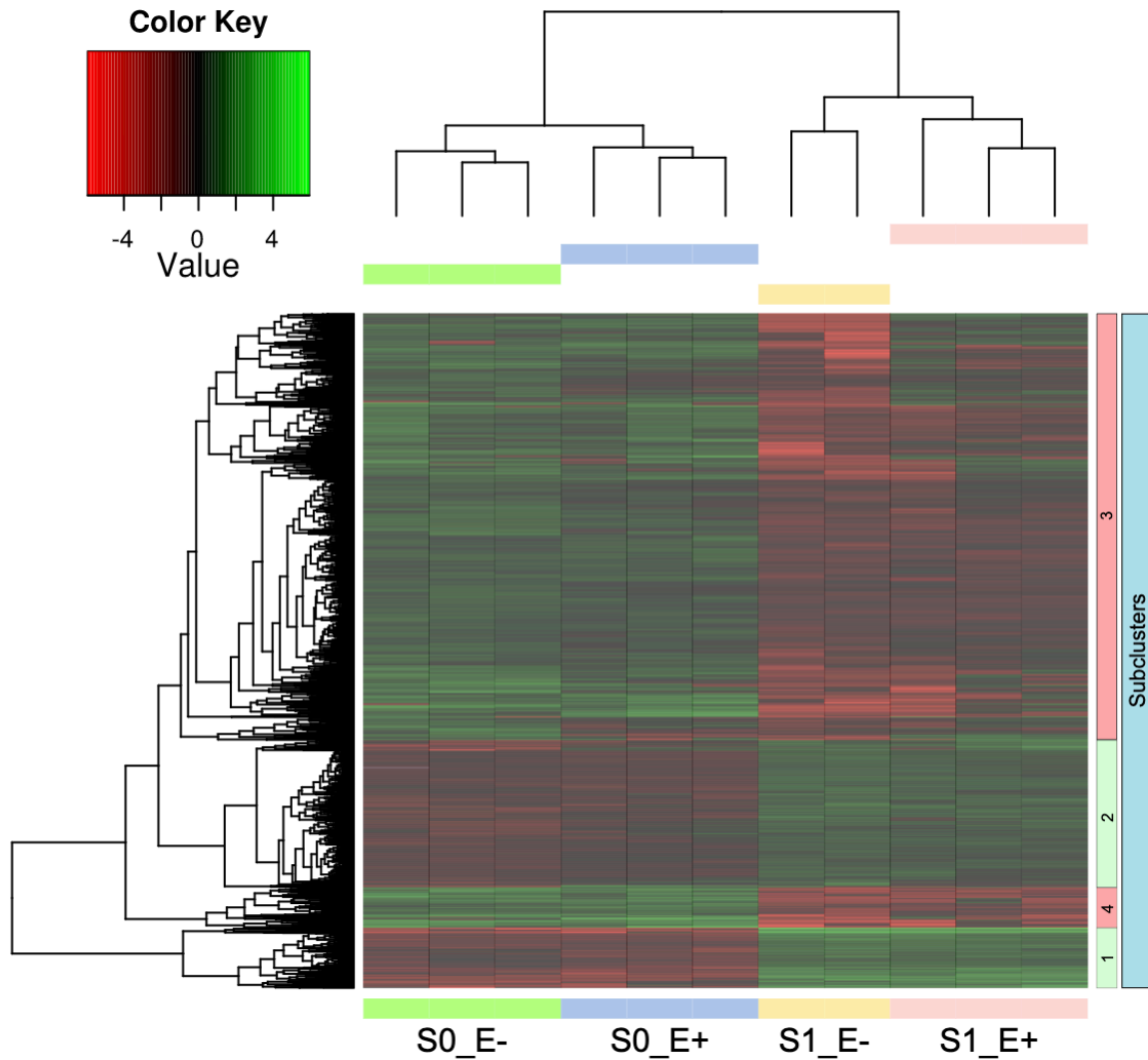
Figure 4.2 – Heatmap showing differential gene expression due to salinity and symbiosis with *Epichloë*. The distribution of the genes is based on the hierarchy of functional classes. X-axis represents different samples from each treatment, [S0_E−]: 0 mM NaCl and without *Epichloë*, [S0_E+]: 0 mM NaCl and infected with *Epichloë*, [S1_E−]: 600 mM NaCl and without *Epichloë*, [S1_E+]: 600 mM NaCl and infected with *Epichloë*. The Y-axis represents the differentially expressed genes. The gene expression is indicated by color, with green represents up-regulated genes, and red the down-regulated genes. The genes were assigned to four expression profile groups: S1 and S2 contained genes up-regulated by salt, and S3 and S4 contained genes down-regulates by salt.
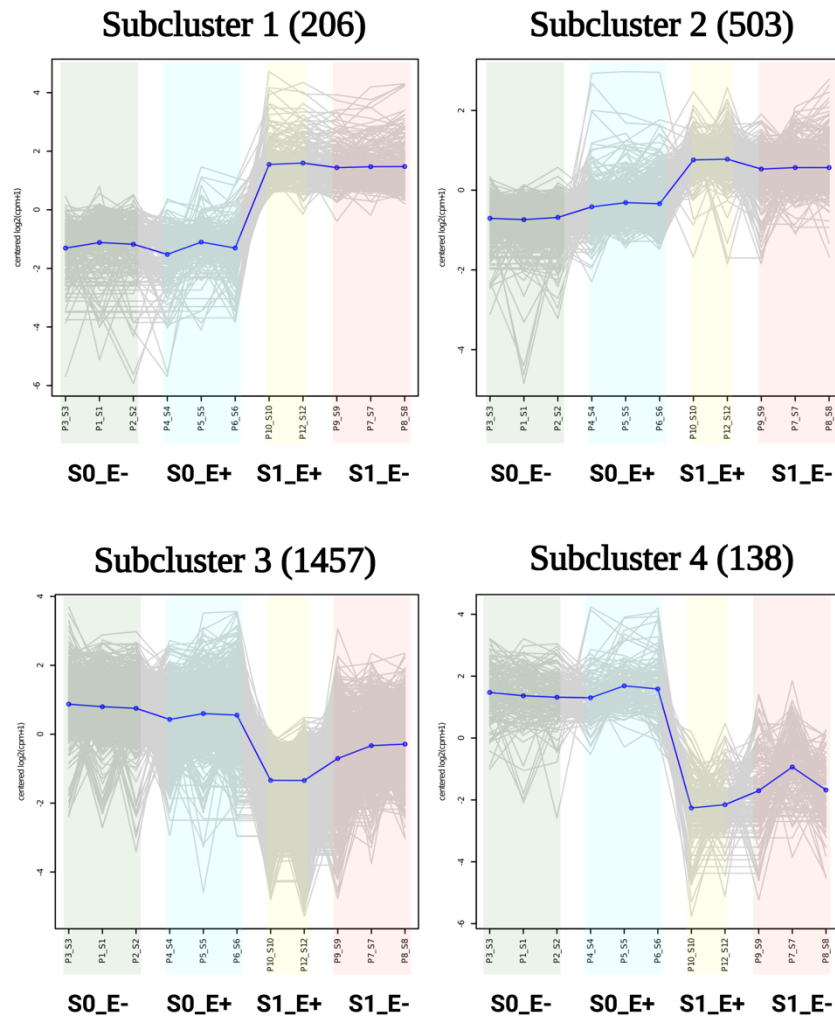
Figure 4.3 – Clustering of the differentially expressed genes (DEGs) based on their expression patterns in response to salinity. Four subclusters comprising 2304 DEGs are exhibited here, the numbers in parentheses indicate DEGs in the corresponding subcluster. X-axis represents different samples from each treatment, [S0_E−]: 0 mM NaCl and without *Epichloë*, [S0_E+]: 0 mM NaCl and infected with *Epichloë*, [S1_E−]: 600 mM NaCl and without *Epichloë*, [S1_E+]: 600 mM NaCl and infected with *Epichloë*; Y-axis represents centralized and normalized expression value. The blue line represents the mean expression trend of DEGs (gray lines) belonging to each subcluster.

4.3.3. Gene ontology analysis

Non-redundant GO-term data were collected for each protein sequence from InterProScan outputs using custom scripts. The GO domains of each subcluster and the number of genes involved are shown in Table 4.2. Regarding the principal Gene Ontology categories, the greatest number of genes in the Biological Process category for Subcluster 1 and 2 belonged to the *oxidation-reduction process* and *metabolomic process*. Subcluster 3 and 4 belonged to *protein phosphorylation* and *oxidation-reduction process*. In the category of Cellular Components compromised *integral component of membrane* and *membrane* for the four

subclusters, while the Molecular Function category consisted mainly of *catalytic activity*, *ATP binding* and *ATPase activity* in the Subcluster 1 and 2, and *ATP binding* and *protein kinase activity* in the Subclusters 3 and 4.

Table 4.2 – Gene Ontology domains and number of genes of each subcluster of Salt-*Epichloë* combination. The expression profile groups Subcluster 1 and Subcluster 2 contained genes up-regulated by salt, while Subcluster 3 and Subcluster 4 contained genes down-regulated by salt.

Ontology domain	Subcluster 1		Subcluster 2		Subcluster 3		Subcluster 4	
	No. of genes	GO-terms	No. of genes	GO-terms	No. of genes	GO-terms	No. of genes	GO-terms
Biological process	149	59	331	110	872	209	88	31
Cellular component	45	12	87	24	350	48	35	10
Molecular function	244	109	547	172	1604	303	170	58
Total	438	180	965	306	2826	560	293	99

Based on the DEGs list, we conducted GO enrichment analysis. The DEGs found in each subcluster were classified into Biological Processes associated with the FRP salt response. A REVIGO analysis (Supek et al., 2011) (Figure 4.4) and the gene hierarchical interaction analysis using OmicsBox (v1.4) (Figure 4.5A,C) for each gene expression group of the Biological Process was performed. Subclusters 1 and 2 revealed GO-terms associated with salt stress regulation, such as proline biosynthesis, metabolic process and ion transport (Figures 4.4 and 4.5A,B4.5A,B). On the other hand, Subcluster 3 revealed salt induced changes in the GO terms associated with photosynthesis and the synthesis of tetrapyrroles, precursors of chlorophyll biosynthesis (Figure 4.4); and Subcluster 4 also revealed GO terms associated with the protein metabolism change induced by salinity (Figures 4.4 and 4.5C). A list of all DEGs with their biological processes and *p*-value are provide in Supplementary Table S4.1.
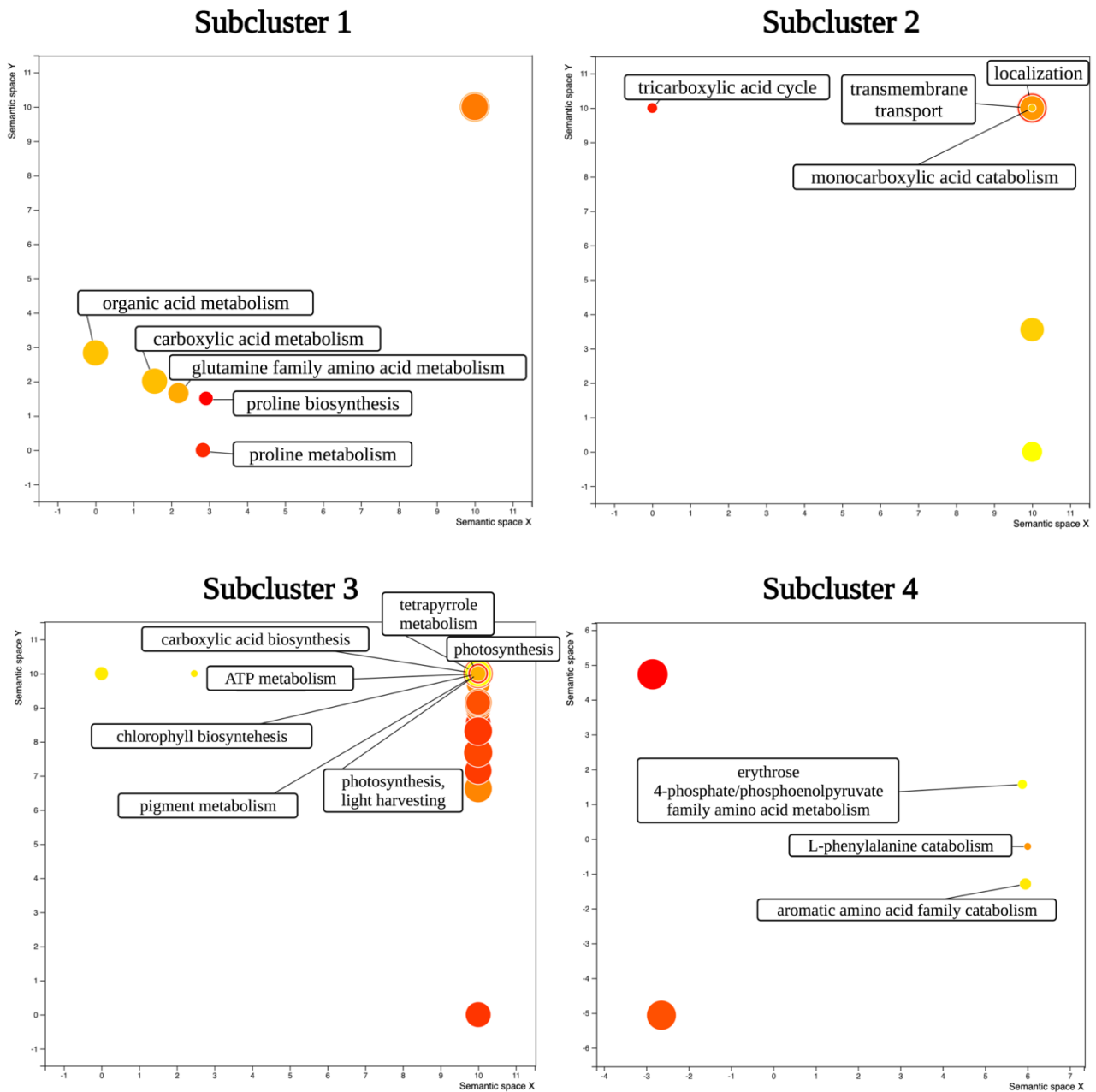
Figure 4.4 – Revigo visualization of biological process gene ontology (GO) term enrichment analysis of differentially expressed genes (DEGs). The proximity of terms represents their semantic similarities and size bubble represents the frequency of the GO term in the underlying protein annotation database database (larger bubbles implies more general terms and lower bubbles implies more specific terms). The color represents the p-value (red higher, yellow lower). Subcluster 1 and Subcluster 2 contained genes up-regulated by salt, while Subcluster 3 and Subcluster 4 contained genes down-regulated by salt. In each subcluster is described the functions of the principal DEGs is described.
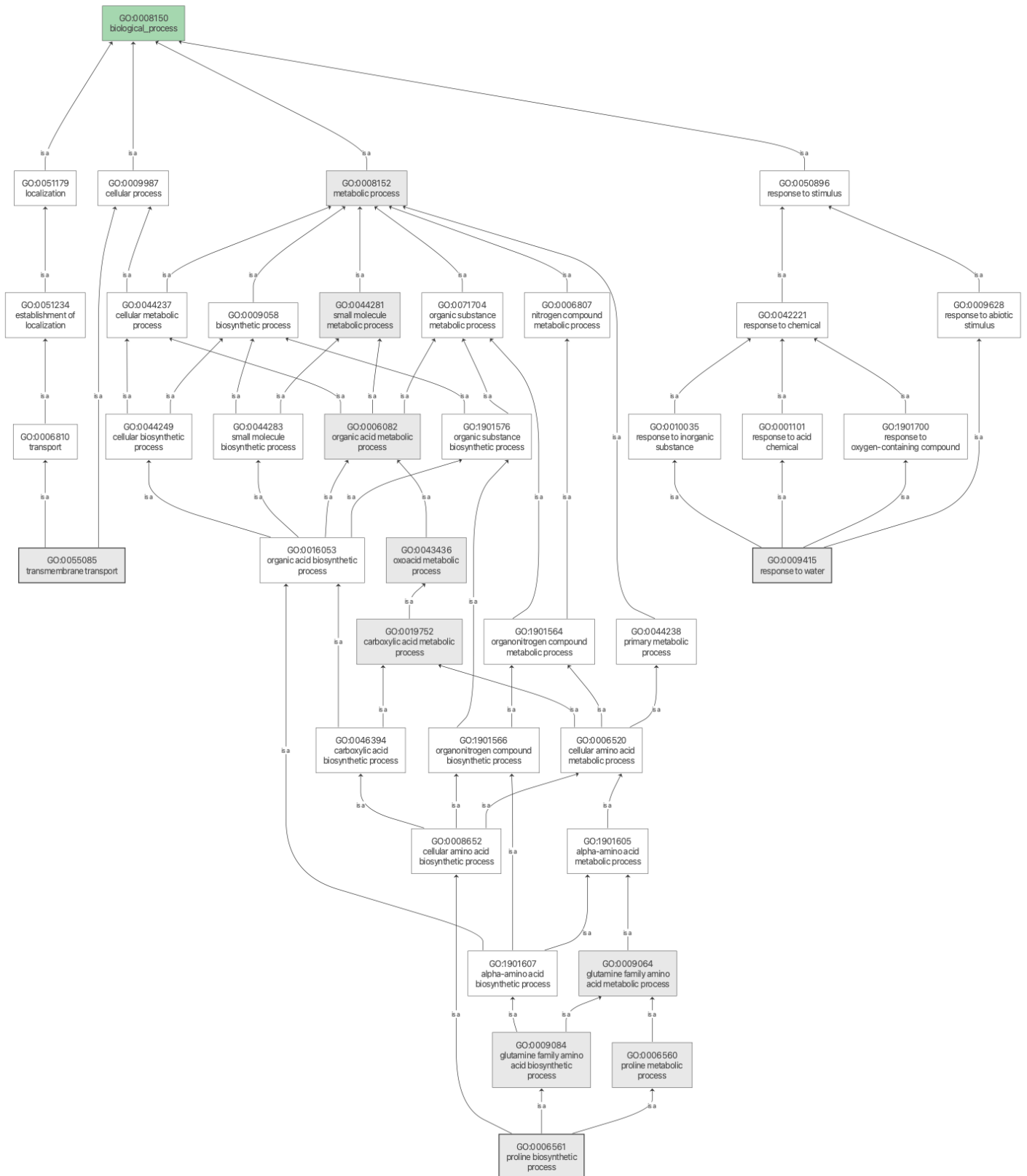
Figure 4.5A – Gene ontology (GO) hierarchical interaction analysis of the Biological Process category in the Subcluster 1 performed in OmicsBox. Each box shows the GO term number and the function. The significant terms are marked with gray color. Subcluster 1 contained genes up-regulated by salt.
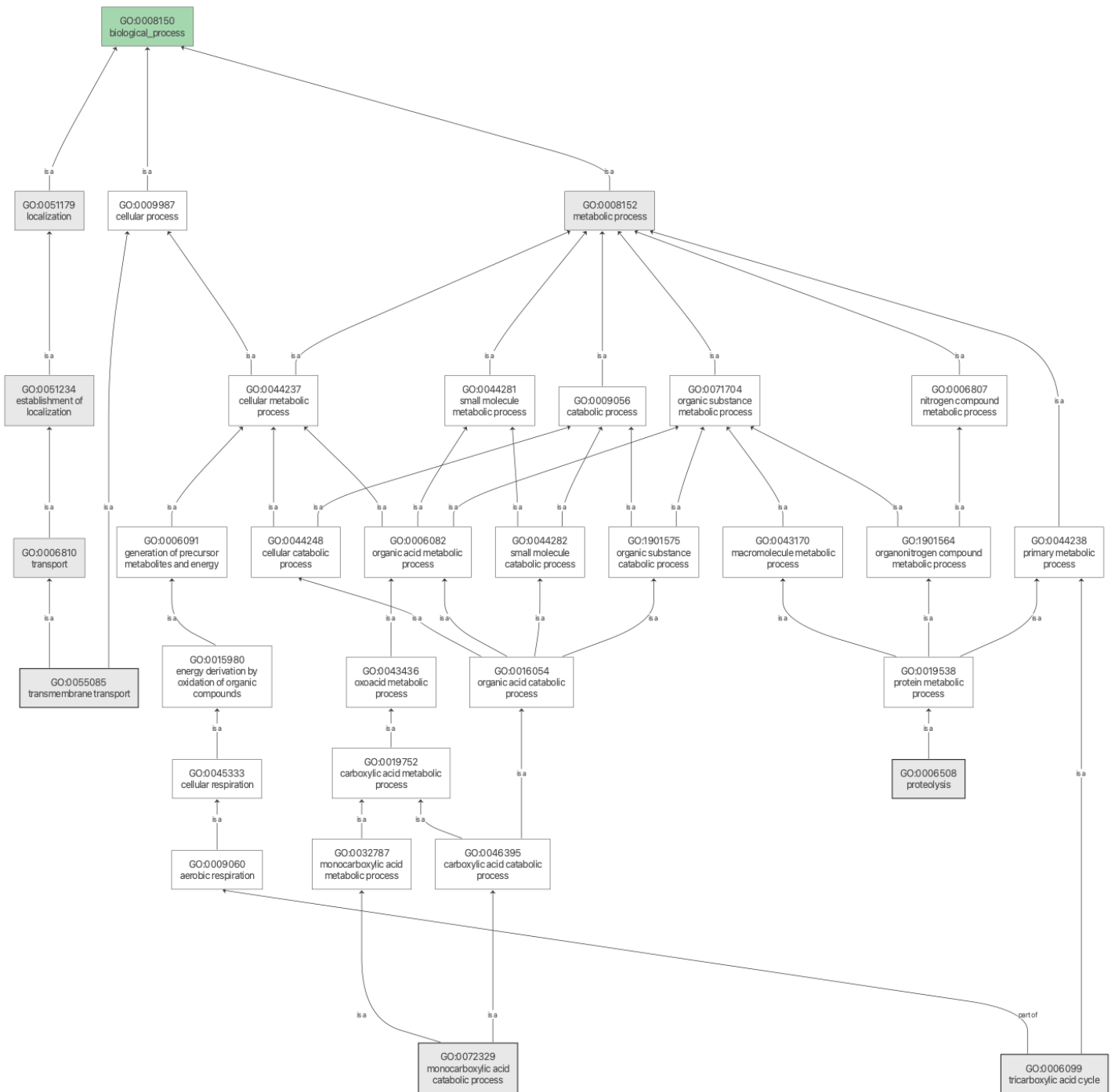
Figure 4.5B – Gene ontology (GO) hierarchical interaction analysis of the Biological Process category in the Subcluster 2 performed in OmicsBox. Each box shows the GO term number and the function. The significant terms are marked with gray color. Subcluster 2 contained genes up-regulated by salt.
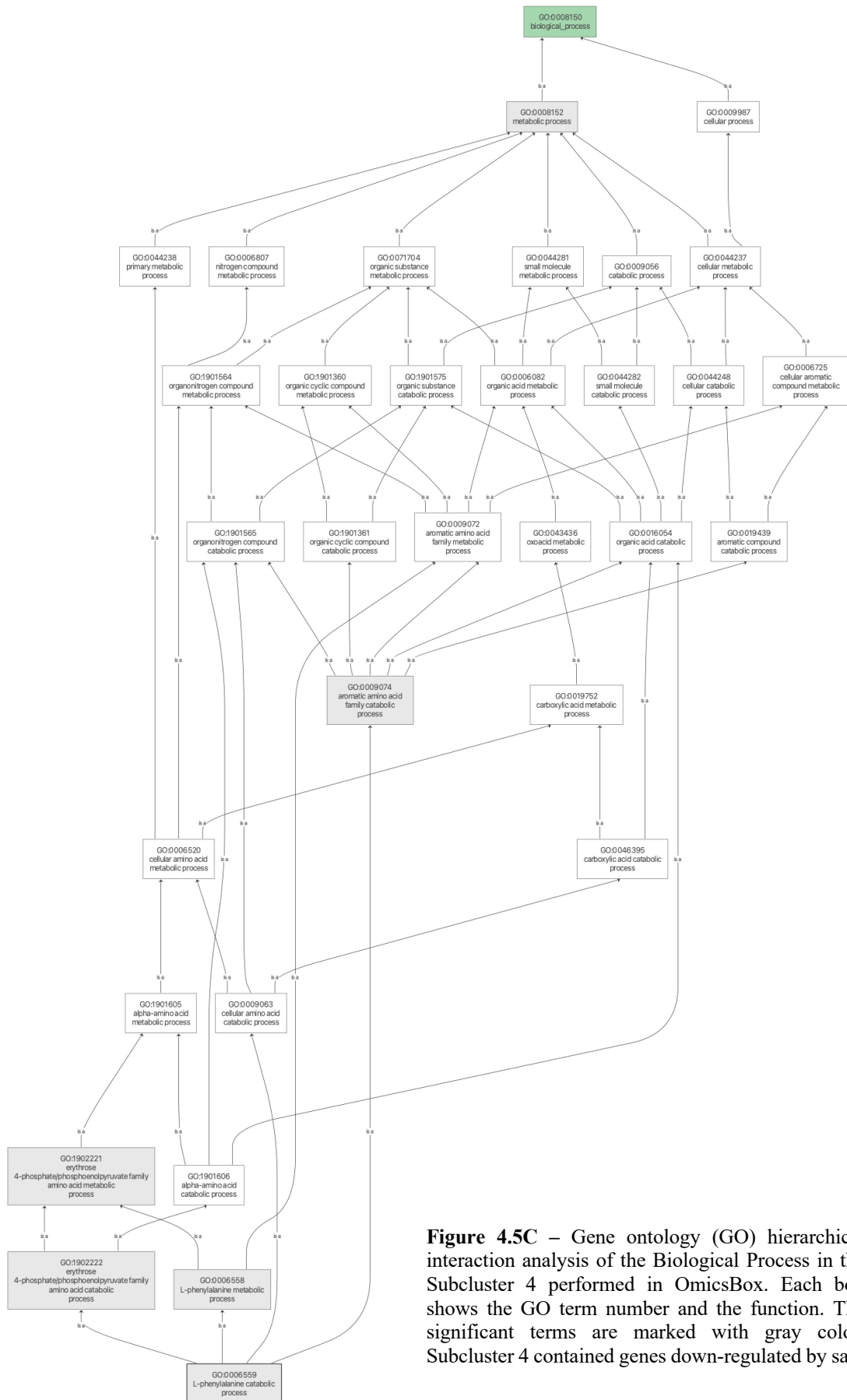
Figure 4.5C – Gene ontology (GO) hierarchical interaction analysis of the Biological Process in the Subcluster 4 performed in OmicsBox. Each box shows the GO term number and the function. The significant terms are marked with gray color. Subcluster 4 contained genes down-regulated by salt.

4.3.4. Untargeted metabolomic analysis of *Festuca* leaves

A total of 130 ions were observed in the set of the methanolic extracts of leaves. The LC-MS profiles showed a tight similarity between all three replicates under the same treatment. Interestingly, two strikingly high ion peaks were observed in extracts from E+ plants, between 4−5 min, and independently of the salt regime (Figure 4.6).
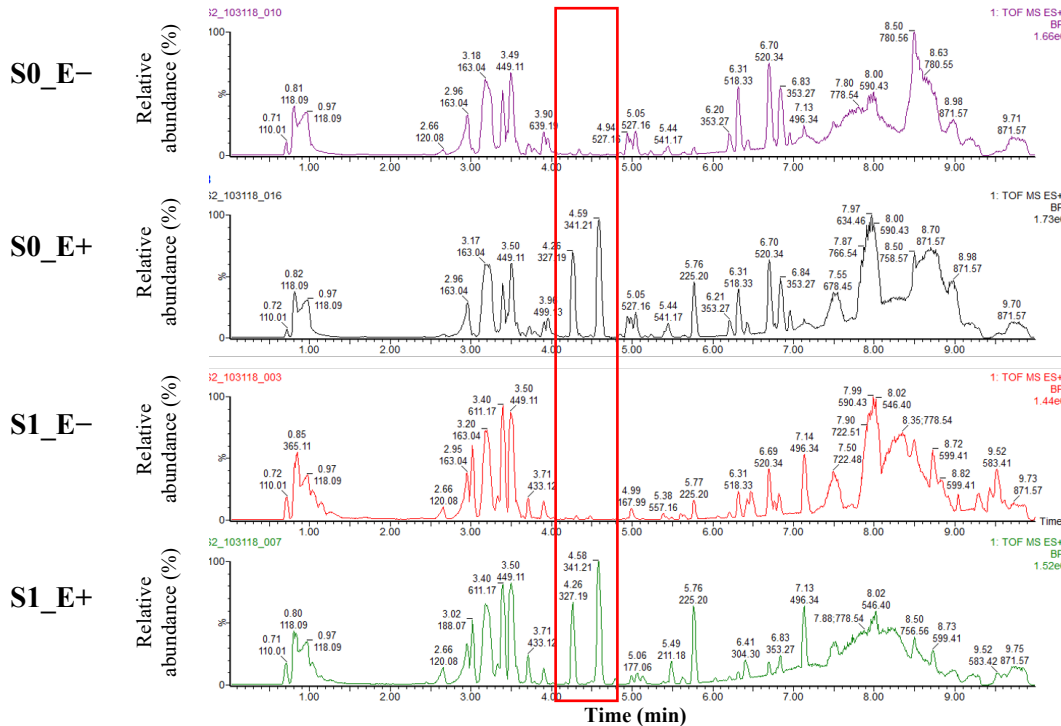
Figure 4.6 – LC-MS chromatogram of untargeted metabolomic analysis of leaves extracts of each treatment. [S0_E−]: 0 mM NaCl and without *Epichloë*, [S0_E+]: 0 mM NaCl and infected with *Epichloë*, [S1_E−]: 600 mM NaCl and without *Epichloë*, [S1_E+]: 600 mM NaCl and infected with *Epichloë*. The red box highlights the two peaks abundant in the symbiotic plants.

In order to evaluate the classes of the ions, the relative mass defect (RMD) values, which measure the fractional hydrogen content of a detected ion, were calculated. This supports the classification of the ions based on the metabolite biosynthetic origin (Ekanayaka et al., 2015). Most of the ions detected in all treatments ranged between 0−850 ppm (Figure 4.7). A high peak of ions with RMD that seems to correspond with terpenoids (700−750 ppm) (Ekanayaka et al., 2015) was observed in all treatments.
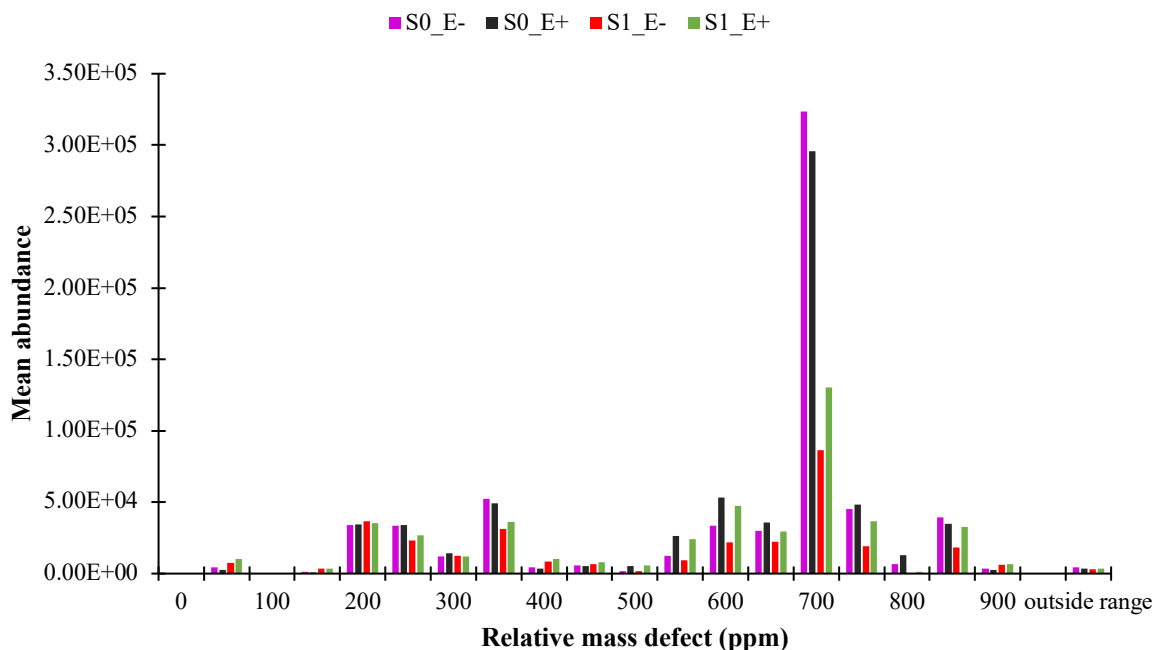
Figure 4.7 – Abundance-weighted histograms of all ions binned by relative mass defect (RMD) at each treatment. [S0_E−]: 0 mM NaCl and without *Epichloë*, [S0_E+]: 0 mM NaCl and infected with *Epichloë*, [S1_E−]: 600 mM NaCl and without *Epichloë*, [S1_E+]: 600 mM NaCl and infected with *Epichloë*.

While profiles of both E− and E+ extracts included similar ranges of ions as described above, two strikingly high ion peaks were observed in extracts from E+ plants, at 4.26 min and 4.58 min regardless of the salt regime (Figure 4.8). These two compounds, *m/z* 327.1922 and *m/z* 341.2086 were distinctly abundant ions in extracts from E+ plants (Figure 4.9) with relative abundance levels of more than 70,000 and 130,000, respectively, and more than fifty-fold higher than the same ions in extracts from E− plants.
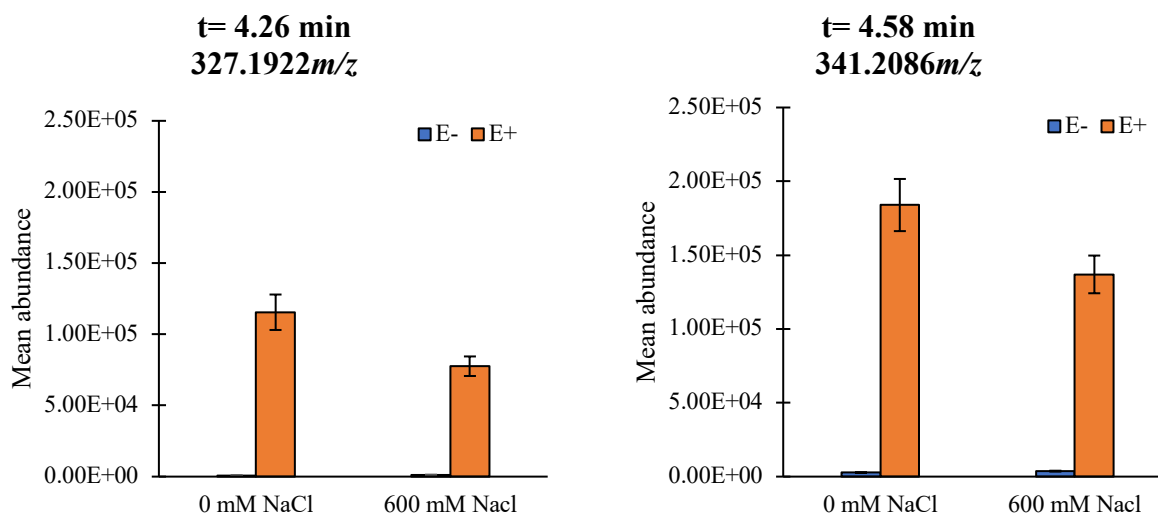
Figure 4.8 – The two abundant ions detected in plants infected with *Epichloë festucae,* indicating the retention time and the mass of each peak.

These two ions had a RDM of 587 ppm and 611 ppm, respectively. The MS spectrum (Figure 4.9) of products of m/z 327.19 (RDM= 588 ppm) with predicted elemental formula $C_{16}H_{27}N_2O_5$, revealed a fragment at m/z 325.1768 (RDM= 528 ppm) that is the [M-H]−ion based on the presence of m/z 371.18 (M+formate) and m/z 361.15 (M+Cl). There is a neutral loss of m/z 166.101 (325.177−159.076) that may correspond to $C_{10}H_{14}O_2$. The 159.076 and 88.039 ions have masses that are consistent with that of a di-alanine peptide fragment (m/z 159.07), which further fragments to lose an alanine (neutral loss of 71.03) and give the m/z 88.039 ion (M-H ion of alanine).
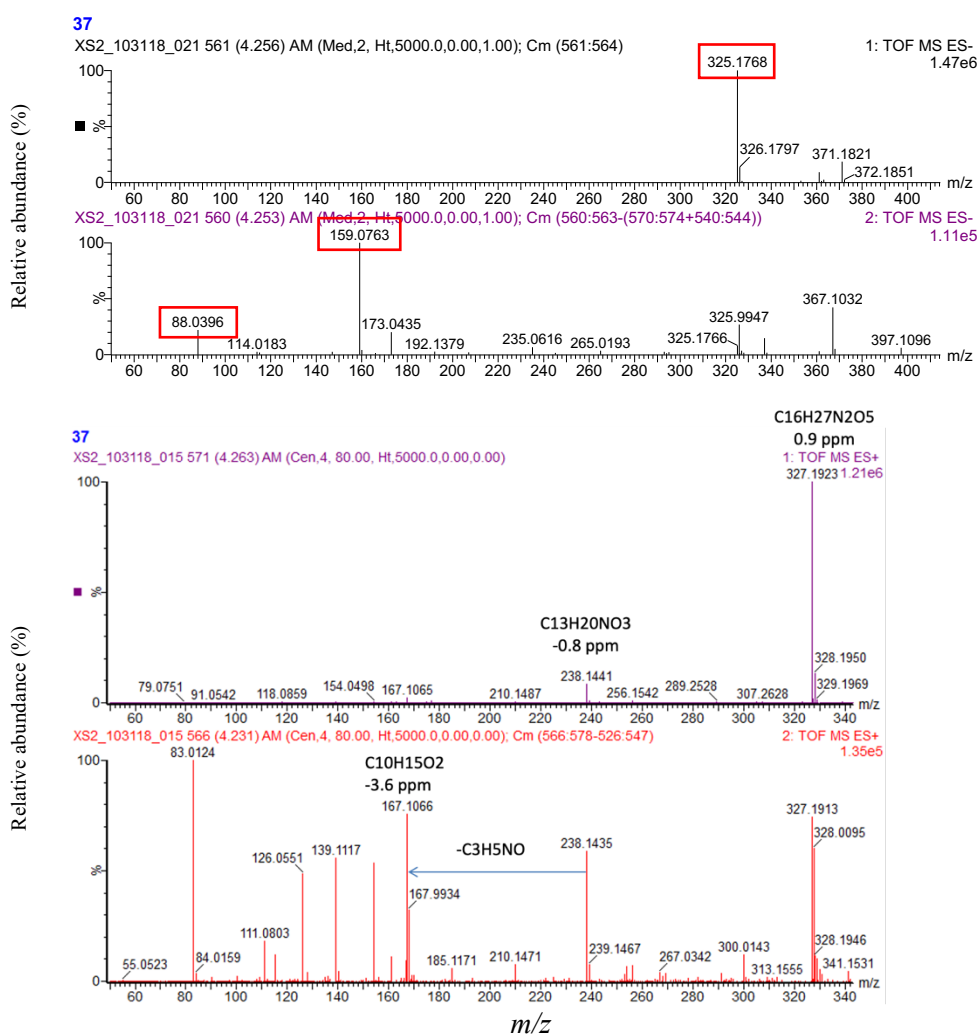
Figure 4.9 – MS spectrum of products of m/z 327.1922 (RMD= 588 ppm) with predicted elemental formula.

In the MS spectrum (Figure 4.10) of products of m/z 341.21 (RDM= 611 ppm) with a predicted elemental formula $C_{17}H_{29}N_2O_5$, revealed the most abundant fragment ion at m/z 339.1934 (RDM= 570 ppm) that is the [M-H]-ion based on the presence of m/z 385.19

(M+formate). In this case, the neutral loss of *m/z* is 180.117 (339.193−159.076). As in the first compound again the fragments *m/z* 159.076 and *m/z* 88.039 ions have masses that are consistent with that of a di-alanine peptide fragment (*m/z* 159.07), which further fragments to lose an alanine (neutral loss of 71.03) and give the *m/z* 88.039 ion (M-H ion of alanine).
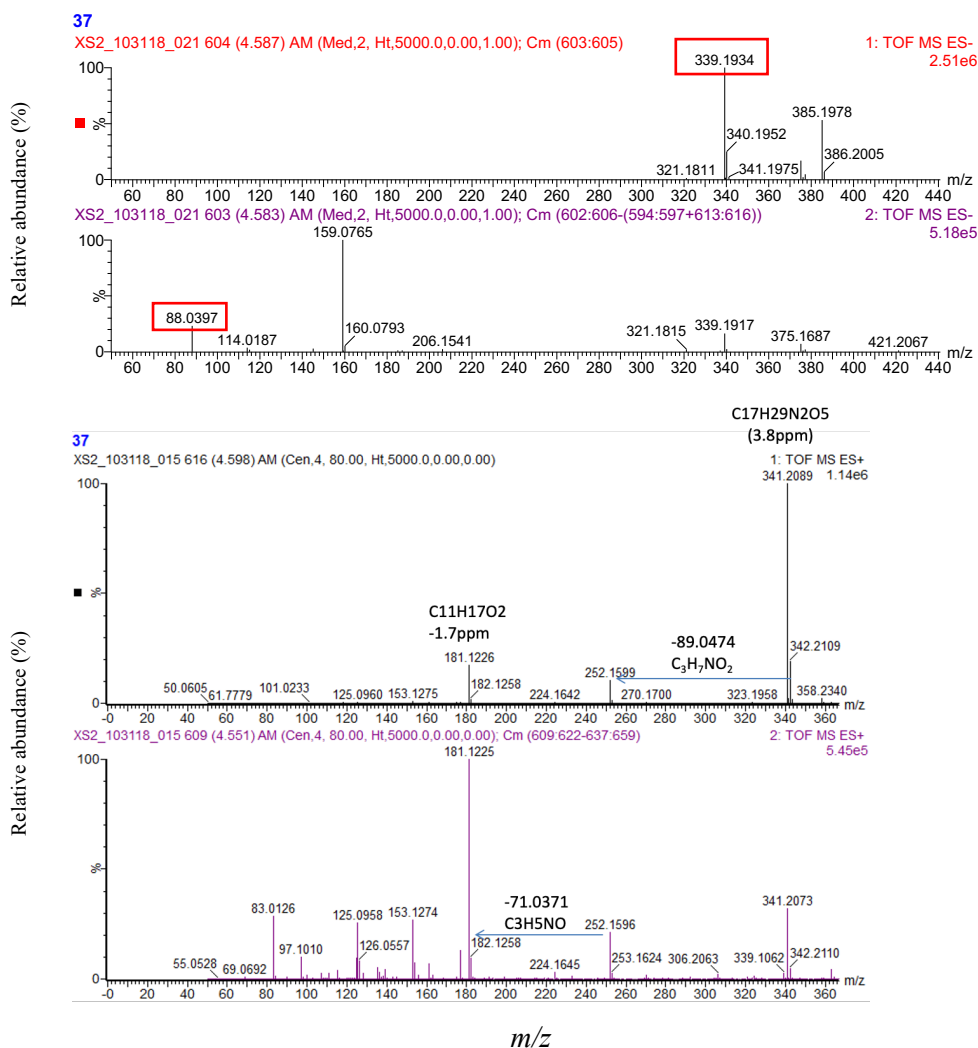
Figure 4.10 – MS spectrum of products of *m/z* 341.2086 (RMD= 611 ppm) with predicted elemental formula.

The information collected with the present data is not sufficient to identify both compounds. However, both compounds appear to be made up of dialanine fragments and contain a still unidentified part.

4.4. DISCUSSION

In the current study, we performed an RNA-seq analysis to investigate transcriptome changes in FRP leaves in response to salinity and also the presence of *E. festucae*. No genome sequence of FRP is presently available, thus we used the *Lolium perenne* gene annotations (Nagy et al., in preparation) as a reference. The main number of DEGs identified were salinity-related components. Interestingly, these changes in gene expression did not seem to be affected by *E. festucae*. The PCA of the transcriptome data showed that salinity was a major factor differentiating the samples, while the variance caused by *Epichloë* was very low. FRP is well adapted to a maritime habitat, however, according to the results shown in the previous chapter of this thesis, *Epichloë* did not seem to have an obvious role in FRP growth and survival under salinity.

Salinity induces damage primarily through osmotic and ion stress. Halophytic plants can resist salt stress due to their ability to adjust osmotic state and ion balance (Flowers and Colmer, 2008; Munns and Tester, 2008; Zhao et al., 2020). In Chapter 3 FRP plants treated with 600 mM NaCl showed a substantial increase in their leaf Na^+ and proline content. Vacuoles appear to sequester excess Na^+ and when this occurs an osmotic adjustment of the cytoplasm is needed to maintain cell turgor, and this was achieved by the accumulation of proline. This balance with proline in FRP plants could be confirmed by our transcriptomic data, which showed an increase in the expression of genes involved in proline synthesis in plants subjected to salinity, such as glutamine family amino acid metabolic process, glutamine family amino acid biosynthetic process, proline metabolic process and proline biosynthetic process. As observed in Chapter 3, this balance with proline occurred in FRP regardless of the presence of *Epichloë*. Furthermore, the expression of transporters and transmembrane transporters was also induced by salt treatment. It is presumed that transporters play crucial roles in maintaining and re-establishing ion homeostasis in halophytes (van Zelm et al., 2020; Zhao et al., 2020). The regulation of the ion transmembrane transport in cells mainly involves the compartmentalization of ions into the vacuole and the influx of ions as K^+ (Shabala and Mackay, 2011). Although in this work we have focused on biological processes, DEGs that belong to Molecular Function category involved in ion transport and expressed by salinity were identified. This is the case of ATPase activity, coupled to the movement of substances and transmembrane movement of substances. Therefore, since FRP leaves exploit Na^+ accumulation and sequestration mechanisms for adaptation to salinity, these transporters would

play roles to transport excess ions to the vacuole and/or to transport K^+ to the cytoplasm. Taken together, these results suggest a regulatory network to control salt acclimatization similar to that reported by Diédhiou et al. (2009) in *F. rubra* subsp. *litoralis*, where a transcriptome analysis also revealed an expression of transcripts involved in proline synthesis and the vacuolar ion transport system (V-ATPase) to control Na^+ homeostasis. Additionally, another transcriptome analysis with the halophyte grass *Sporobolus virginicus* also showed an osmotic adjustment in adaptation to salt stress and an increase in the expression of genes involved in proline biosynthesis and ion transporters (Yamamoto et al., 2015).

One of the most noticeable impacts of the salinity treatment was a decrease in the expression of genes related to photosynthesis, particularly the light reaction. For example, our transcriptomic data showed that the expression of genes involved in light harvesting, such as photosynthesis light-harvesting, chlorophyll metabolic process, chlorophyll biosynthetic process, pigment biosynthetic process, pigment metabolic process decreased with salinity. One of the consequences of salinity-induced photo-synthetic impairment is the exposure of the plant to an excess of light, which inevitably provokes the photodamage to photosystem II (Duarte et al., 2014). Plants under salt stress use less light energy for photosynthesis and the absorbed light energy cannot be used productively. Such energy imbalance leads to an overexcitation of the photosynthetic apparatus that in turn increases the potential for photoinhibition and subsequent photooxidative damage (Chaves et al., 2009; Wungrampha et al., 2018). To avoid the energy imbalance resulting from salinity, FRP plants seem to down-regulate genes associated with photosynthetic light-harvesting to reduce light energy absorption. This seems to be consistent with previous studies (Gharat et al., 2016; Zhang et al., 2020). Consequently, this down-regulation affects all processes dependent on photosynthesis. For example, the rate of ATP synthesis depends on the light reaction, thus a decrease in light-harvesting affects the ATP cycle. Our datasets revealed a decrease in the expression of genes related to the ADP metabolic process, ATP generation from ADP. Also, a decrease in the gene expression involved with the shikimate pathway was detected, as erythrose 4−phosphate/phosphoenolpyruvate family amino acid metabolism, L−phenylalanine catabolism and aromatic amino acid family catabolism. The salinity decreased photosynthesis and may cause a reduced production of reducing force and limited nitrogen metabolism, which in turn reduces the production of amino acids and inhibits glycolysis. The shikimate pathway initiates from erythrose 4−phosphate (E−4P) and phosphoenolpyruvate (PEP) and comprises seven reactions catalyzed by six enzymes to produce chorismite, the precursor of the aromatic amino acids. E−4P and PEP are respectively derived from the non-oxidative branch of the

pentose phosphate pathways and glycolysis, thus connecting the shikimate pathway to central carbon metabolism (Tzin and Galili, 2010; Maeda and Dudareva, 2012). Therefore, a reduction in the photosynthesis expression and consequently in glycolysis could be involved in the down-expression of this pathway.

An untargeted metabolomic analysis was performed to investigate the metabolic changes that salinity and *Epichloë* can produce in FRP leaves. The FRP extract profiles were similar among treatments, and based on relative mass defect (RMD) the majority of ions detected corresponded to terpenoids. This result is not surprising because plants can synthesize a suite of several hundred terpenoid compounds with roles that include phytohormones, protein modification reagents, antioxidants, and more (Pichersky and Raguso, 2016). Besides, terpenoids are abundant and widely diverse in halophyte grasses (Faustino et al., 2019).

A remarkable increase in the abundance of two ions occurred in plants infected with *Epichloë*. Interestingly this increase was independent of the salinity regime, suggesting that these compounds have no role in the plant adaptation to salt stress. Although these two ions had RMDs consistent with terpenoids, their identification was not possible. Both compounds are constituted by fragments of dialanine, but contain a still unidentified part. For a correct and conclusive identification additional analyses are necessary. *Epichloë* endophytes can induce several changes in the metabolic profiles of grasses because of their capacity to produce several classes of biologically active alkaloids that provide selective benefits to the host plants (Schardl et al., 2004; 2013; Saikkonnen et al., 2016). Nevertheless, in this case, they are not metabolites produced by *Epichloë* because both were present in E− plants, although at a much lower level. Therefore, they seem to be compounds potentialized by the presence of the fungus. It has been suggested that *Epichloë* may play important roles inducing host compounds, such as volatile organic compounds (Li et al., 2014), phenolic compounds (Qawasmeh et al., 2012a; 2012b), or plant metabolites in root exudates (Patchett and Newman, 2021), and changes in primary metabolites (Rasmussen et al., 2008; 2012; Dupont et al., 2015; Green et al., 2020).

Epichloë endophytes are known to produce bioactive alkaloids, as ergovaline and indole diterpenes which including lolitrem B (Repussard et al., 2014; Guerre, 2015; 2016; Vázquez de Aldana et al., 2020). It is known that the alkaloid ergovaline is produced by *E. festucae* in FRP (Vázquez de Aldana et al., 2007). However, no peak corresponding to ergovaline was identified in the present work. Phenotypic variation in ergovaline can occur among symbiotic FRP plants (Vázquez de Aldana et al., 2007), and the absence of ergot alkaloids in symbiotic plants may be due to the absence of functional genes from the biosynthetic pathway (Schardl et al., 2014). However, in a previous study we found that the *E. festucae* strain (CD8) used for

this work contained all the genes required for the synthesis of ergovaline, whose presence was verified by HPLC (Thon et al., 2018). Therefore, the absence of ergovaline in the present analysis may be related to the different extraction method used or to the quantification parameters. In a study developed by Vassiliadis et al. (2019), ergovaline was quantified using LC–MS but several quantification parameters had to be adjusted.

4.5. CONCLUSIONS

In this study, transcriptomic and metabolomic analyses were carried out to gain insight into the salt stress response of *Festuca rubra* subsp. *pruinosa* and the role of symbiosis with *Epichloë festucae* in this response. The transcriptome analysis confirmed some mechanisms used by *Festuca rubra* to cope with salinity, which were described in Chapter 3. Increased expression of genes involved in ion transport and proline biosynthesis seems to be related to a tissue tolerance mechanism that accumulates Na^+ in vacuoles and uses proline for an osmotic counterbalance in the cytoplasm. Additionally, *Festuca rubra* down-regulated genes associated with photosynthetic light-harvesting processes, possibly to prevent photoinhibition and subsequent photooxidative damage.

The untargeted metabolomic analysis showed that *Festuca rubra* extract profiles were similar among treatments, and based on relative mass defects the majority of ions detected corresponded to terpenoids. In this case, an obvious effect of *Epichloë* was observed in the abundance of two compounds regardless of the salinity regime that decreased drastically in the absence of *Epichloë*. Unfortunately, the information collected was not sufficient to reach a conclusive identification of these two compounds.

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4.7. SUPPLEMENTARY MATERIAL

Supplementary Table S4.1 – List of differentially expressed genes (DEG) in each subcluster classified into Biological Process category. Subcluster 1 and Subcluster 2 contained genes up-regulated by salt, while Subcluster 3 and Subcluster 4 contained genes down-regulated by salt.

Biological process, Subcluster 1

Term ID	Description	Frequency (%)	$\log_{10}$ p-value
GO:0008150	biological process	100	-5.6868
GO:0008152	metabolic process	75.387	-4.7282
GO:0044281	small molecule metabolic process	15.138	-4.5696
GO:0055114	oxidation-reduction process	15.06	-4.9797
GO:0006082	organic acid metabolic process	9.086	-4.186
GO:0055085	transmembrane transport	8.916	-4.1712
GO:0019752	carboxylic acid metabolic process	8.831	-4.2357
GO:0009064	glutamine family amino acid metabolic process	1.157	-4.4303
GO:0006560	proline metabolic process	0.187	-6.0507
GO:0006561	proline biosynthetic process	0.163	-6.6508
GO:0009415	response to water	0.026	-3.6272

Biological process, Subcluster 2

Term ID	Description	Frequency (%)	$\log_{10}$ p-value
GO:0008150	biological process	100	-5.3543
GO:0008152	metabolic process	75.387	-5.3194
GO:0090304	nucleic acid metabolic process	21.449	-3.8591
GO:0051179	localization	18.495	-4.6054
GO:0006810	transport	17.616	-4.6772
GO:0055114	oxidation-reduction process	15.06	-4.2976
GO:0055085	transmembrane transport	8.916	-5.7385
GO:0006508	proteolysis	5.223	-3.5169
GO:0006099	tricarboxylic acid cycle	0.469	-5.4182
GO:0072329	monocarboxylic acid catabolic process	0.290	-3.9556

Biological process, Subcluster 3

Term ID	Description	Frequency (%)	$\log_{10}$ p-value
GO:0008150	biological process	100	-5.1079
GO:0008152	metabolic process	75.387	-5.2231
GO:0009987	cellular process	63.78	-5.2474
GO:0071704	organic substance metabolic process	58.357	-5.1966
GO:0044238	primary metabolic process	53.743	-5.0896
GO:0044237	cellular metabolic process	53.061	-5.2914
GO:0009058	biosynthetic process	31.611	-5.5954

Term ID	Description	Frequency (%)	$\log_{10}$ p-value
GO:1901576	organic substance biosynthetic process	30.365	-5.5243
GO:0044249	cellular biosynthetic process	30.048	-5.5152
GO:0006139	nucleobase-containing compound metabolic process	26.547	-3.933
GO:0090304	nucleic acid metabolic process	21.449	-5.4653
GO:0051179	localization	18.495	-4.0025
GO:1901564	organonitrogen compound metabolic process	17.886	-5.2473
GO:1901362	organic cyclic compound biosynthetic process	17.871	-2.7153
GO:0006810	transport	17.616	-3.9286
GO:0044281	small molecule metabolic process	15.138	-5.5504
GO:0055114	oxidation-reduction process	15.060	-5.4973
GO:0044267	cellular protein metabolic process	14.293	-4.1903
GO:1901566	organonitrogen compound biosynthetic process	14.064	-4.3991
GO:0006793	phosphorus metabolic process	13.507	-5.5669
GO:0006796	phosphate-containing compound metabolic process	13.11	-5.4363
GO:0043412	macromolecule modification	9.785	-4.5778
GO:0006082	organic acid metabolic process	9.086	-4.2721
GO:0055085	transmembrane transport	8.916	-5.5394
GO:1901135	carbohydrate derivative metabolic process	6.319	-3.9907
GO:0044283	small molecule biosynthetic process	5.677	-5.7227
GO:0006259	DNA metabolic process	5.607	-5.6904
GO:0005975	carbohydrate metabolic process	5.26	-5.5643
GO:0046394	carboxylic acid biosynthetic process	4.159	-3.7892
GO:0006468	protein phosphorylation	4.137	-5.3915
GO:1901605	alpha-amino acid metabolic process	3.625	-5.0475
GO:0006629	lipid metabolic process	3.522	-4.7121
GO:0019693	ribose phosphate metabolic process	3.032	-3.1114
GO:0051188	cofactor biosynthetic process	2.763	-3.1686
GO:0072521	purine-containing compound metabolic process	2.673	-3.0733
GO:0032787	monocarboxylic acid metabolic process	2.485	-3.576
GO:0008610	lipid biosynthetic process	2.123	-4.141
GO:0006091	generation of precursor metabolites and energy	1.940	-6.0167
GO:0046034	ATP metabolic process	1.263	-3.8356
GO:1901361	organic cyclic compound catabolic process	1.164	-2.7943
GO:0019439	aromatic compound catabolic process	1.164	-2.8346
GO:0016052	carbohydrate catabolic process	1.078	-4.795
GO:0033013	tetrapyrrole metabolic process	0.834	-6.2733
GO:0006090	pyruvate metabolic process	0.817	-2.677
GO:0046939	nucleotide phosphorylation	0.792	-3.6263
GO:0009072	aromatic amino acid family metabolic process	0.719	-2.9309
GO:0009132	nucleoside diphosphate metabolic process	0.698	-3.5655
GO:0015074	DNA integration	0.682	-5.7708
GO:0019318	hexose metabolic process	0.641	-3.5777
GO:0006165	nucleoside diphosphate phosphorylation	0.600	-3.6263

Term ID	Description	Frequency (%)	log₁₀ p-value
GO:0006511	ubiquitin-dependent protein catabolic process	0.584	-2.6717
GO:1901606	alpha-amino acid catabolic process	0.569	-5.7643
GO:0009069	serine family amino acid metabolic process	0.544	-3.8232
GO:0042440	pigment metabolic process	0.485	-4.1921
GO:0034637	cellular carbohydrate biosynthetic process	0.478	-3.0420
GO:0006544	glycine metabolic process	0.221	-3.4300
GO:0015979	photosynthesis	0.183	-6.4204
GO:1902221	erythrose 4-phosphate/phosphoenolpyruvate family amino acid metabolic process	0.075	-3.5706
GO:0017144	drug metabolic process	0.058	-3.9995
GO:0001505	regulation of neurotransmitter levels	0.055	-2.8812
GO:0015995	chlorophyll biosynthetic process	0.039	-6.7686
GO:0009765	photosynthesis, light harvesting	0.019	-6.6059
GO:0042133	neurotransmitter metabolic process	0.006	-2.8812

Biological process, Subcluster 4

Term ID	Description	Frequency (%)	log₁₀ p-value
GO:0008150	biological process	100	-5.7047
GO:0008152	metabolic process	75.387	-5.1052
GO:0009074	aromatic amino acid family catabolic process	0.116	-4.1537
GO:1902221	erythrose 4-phosphate/phosphoenolpyruvate family amino acid metabolic process	0.075	-4.0257
GO:0006559	L-phenylalanine catabolic process	0.031	-4.6418

Chapter 5

A *Diaporthe* strain promotes tomato growth and enhances drought tolerance

Abstract

Drought stress is a major constraint for plant growth and crop production worldwide. Due to the drastic and rapid changes in the global climate, drought is likely to increase in frequency and severity. Tomato is one of the most important crops in the world and very sensitive to water deficit, so the search for strategies to improve its tolerance or adaptation to drought stress is imperative. Functional symbiosis with microbes like fungal endophytes can help plants to adapt to environmental stress. This study aimed to investigate the ability of a *Diaporthe* endophyte strain isolated from roots of *Festuca rubra* subsp. *pruinosa* to ameliorate the impact of drought stress on tomato plants. Individual plants of tomato inoculated and uninoculated with *Diaporthe* were exposed to limiting water conditions. In response to drought stress, uninoculated plants exhibited a reduction in growth with a concomitant decrease in photosynthetic activity and nutrient uptake. When tomato plants were inoculated with *Diaporthe* growth and biomass were significantly promoted regardless of the water regime, playing an important role in drought stress tolerance. The symbiotic plants improved the photosynthetic activity and water-use efficiency under drought stress, and also showed increased antioxidant enzyme activity, proline content and nutrient uptake. In summary, these results suggest that *Diaporthe* can be a strategic biological agent to take into account against drought stress in tomatoes.

5.1. INTRODUCTION

Agriculture is highly dependent on the climate, and changes in temperature, atmospheric CO_2, or the frequency and intensity of extreme weather can have a significant impact on crop yields. According to the European Environment Agency (2020), most of Europe has experienced increases in near-surface air and land temperature during the last decade. Additionally, the occurrence of drought events has increased in large parts of Europe, particularly in the Mediterranean region (Caloiero et al., 2018).

Drought is a multidimensional stress that causes a wide range of morphophysiological, biochemical and molecular modifications on plants, resulting in a reduction of plant growth and development (Shahzad et al., 2016; Salehi-Lisar and Bakhshayeshan-Agdam, 2016). Water deficit can cause a series of harmful changes in some central processes in plant cells, including disorders in cell water homeostasis, perturbations in cell metabolic functions, hormonal imbalance, chlorophyll synthesis, root differentiation, foliage dimensions, alteration of stomatal movements, water and mineral nutrition association with decreased plant yield and water use efficiency (Hura et al., 2007; Farooq et al., 2009; Salehi-Lisar and Bakhshayeshan-Agdam, 2016; Kapoor et al., 2020; Zhao et al., 2020). Moreover, drought stress also induces the generation of reactive oxygen species (ROS), which cause oxidative stress and disturb the cell redox regulatory functioning (Cruz de Carvalho, 2008; Impa et al., 2012).

Plants have diverse protection mechanisms to combat drought stress conditions, aimed at capturing more water from the soil or minimizing water loss via transpiration (Shao et al., 2008; Aroca and Ruiz-Lozano, 2012; Yadav and Sharma, 2016; Seleiman et al., 2021). For example, plants suffer certain phenological changes, as a decline in growth rate and an increase of root size for a deepening into the soil in order to absorb the maximum amount of water from deep soil (Hund et al., 2009; Salehi-Lisar and Bakhshayeshan-Agdam, 2016). In response to drought stress, the stomatal closure reduces transpirational water loss, but this also causes a decrease in both CO_2 diffusion and photosynthetic carbon assimilation rate (Shahzad et al., 2016). The overproduction of compatible organic solutes, as proline, is one of the most common mechanisms to protect plants, contributing to osmotic adjustment, detoxification of ROS, and stabilization of membranes, enzymes and protein structures (Farooq et al., 2009). In order to cope with oxidative stress, plants also use an antioxidant defense system, which can be either enzymatic or non-enzymatic (Shazad et al., 2016). This antioxidant apparatus helps in ROS scavenging and the regeneration of ascorbate (AsA) using enzymatic antioxidants such

as catalase (CAT), ascorbate peroxidase (APX) or dehydroascorbate reductase (DHAR) (Noctor et al., 2014; Laxa et al., 2019).

Tomato (*Solanum lycopersicum* L.) is one of the most important crops in the world. In addition to its great economic value, this crop also has a great nutritional value (Story et al., 2010; Raiola et al., 2014). The European Union is among the top five tomato producers worldwide and countries such as Italy, Spain and Portugal are leading producers (Costa and Heuvelink, 2018). Although these countries have the most favorable conditions for tomato production, they are also more conducive to water limitation that has worsened with the climate changes. The high sensitivity of tomato to water deficit has prompted different approaches for breeding drought-resistant crops, including the search for strategies to make them more tolerant or better adapted to drought.

It is essential to search for economically and environmentally efficient strategies to facilitate crop production under drought stress. Microbes could be a promising solution for a sustainable and environment-friendly agriculture due to their ability to promote plant growth, and increase biotic and abiotic stress resistance, recycle nutrients, manage soil fertility and other abilities that can result in the reduced use of fertilizers and pesticides (Meena et al., 2017; Ray et al., 2020; Vishwakarma et al., 2020). Endophytes are symbiotic microorganisms that successfully colonize the internal tissues of plants without causing deleterious symptoms (Hardoim et al., 2015). This symbiotic relationship can confer mechanisms to perform or modulate various and essential functions in plants that directly affect growth, development and resistance to drought stress conditions (Dastogeer and Wylie, 2017; Morsy et al., 2020; Mathur and Roy, 2021).

Diaporthe is one of the most abundant fungal endophytes associated with the roots of *Festuca rubra* subsp. *pruinosa* (FRP), a grass that grows in sea cliffs. A *Diaporthe* strain isolated from this grass ameliorated the salt stress response in *Lolium perenne* and tritordeum plants (Chapter 2, Pereira et al., 2019; Vázquez de Aldana et al., 2019). Symbiotic microorganisms adapted to salinity stress might be of potential benefit to plants in their adaption to drought stress (Rodriguez et al., 2008). Therefore, these characteristics could make *Diaporthe* a promising candidate to improve plant adaptation to drought stress.

This work aimed to evaluate the effect of a *Diaporthe* sp. strain in the growth and physiology of tomato plants under low water availability, focusing on biomass, plant-water relations, photosynthesis, metabolite accumulation, oxidative stress response and nutrient uptake.

5.2. MATERIALS AND METHODS

5.2.1. Fungal strain

The *Diaporthe* strain EB4 was isolated from roots of FRP plants from a natural population in the northern coast of Galicia, Spain (Chapter 2, Pereira et al., 2019). This strain was selected because it belongs to a taxon that is a component of the core microbiome of FRP roots, having an incidence in plants greater than 50% (Chapter 2, Pereira et al., 2019). Fungal mycelium used as inoculum was prepared in beet pulp medium and grown at ambient temperature for four weeks (Vázquez de Aldana et al., 2020).

5.2.2. Experimental design

To determine the effect of *Diaporthe* EB4 on tomato plant growth under drought stress (DS), a bioassay was designed with four treatments, each one with ten replicates: uninoculated, inoculated with *Diaporthe* EB4, uninoculated + DS, and *Diaporthe* + DS. To obtain inoculated plants, seeds of tomato cv. Marmande were sown in a plastic tray containing a substrate composed of seven parts of peat and perlite (1:1 v/v) previously treated at 80 °C for 24 h, and one part of *Diaporthe* EB4 inoculum. To obtain uninoculated plants, seeds were sown in a tray containing only the peat and perlite substrate. Ten-day-old seedlings were then individually transplanted to 300 ml plastic plots containing the substrate with or without inoculum for inoculated and uninoculated seedlings, respectively.

Plants were exposed to a normal watering regime for a week and after this period of adaptation, two watering treatments were applied for five weeks: drought stress and normal water regime. In the drought stress treatment, plants were watered three times per week at 10% of the water holding capacity of the soil. To avoid plant death and thus prolong the treatment, plants were watered once at 100% of the water holding capacity in the middle of the treatment. In the normal water regime, plants were watered three times per week at 100% of the water holding capacity of the soil throughout the treatment. The water holding capacity of the soil was determined by the amount of water held by dry soil sample necessary to fill a pot.

Five weeks after the watering treatment was initiated, all the plants were harvested. From each plant, three leaves from the same branch were pooled and immediately immersed in liquid nitrogen and kept at −80 ºC for antioxidant enzymatic analysis. Then, each plant was

separated into leaves, stems and roots and lyophilized to measure dry weight and for chemical analysis after grinding.

5.2.3. Chlorophyll content

The chlorophyll content was determined 24 h before harvesting by using a leaf-clip sensor (Dualex Force, Orsay, France). In each plant, three leaves of the third branch from the top were selected, and the average chlorophyll content was obtained from three measurements performed at the central position of each leaf.

5.2.4. Photosynthetic parameters

The gas exchange measurements, including stomatal conductance, CO_2 assimilation rate, and water use efficiency (WUE) were obtained using the CIRAS-3 portable gas exchange system (CIRAS−3, PP-Systems, USA) on leaves of the third branch from the top, of four randomly replicate plants per each treatment, 24 h before plant harvesting.

5.2.5. Analysis of nitrogen, phosphorus and potassium content

Five plant replicates of each treatment were analyzed for the N, P and K content. For the analysis, leaves of each plant were pooled into a single sample. Freeze-dried and ground samples were calcined at 450 ºC for 8 h, and ashes were dissolved in $HCl:HNO_3:H_2O$ (1:1:8). Then, the P and K content was determined by inductively coupled plasma atomic emission spectroscopy (ICP−OES) in a Varian 720−ES spectrometer (Agilent, USA). The N content was analyzed by the Dumas combustion method in a C−N analyzer (Leco CHN−628, USA). For each nutrient, total uptake was calculated as the product of the nutrient concentration and the shoot dry weight.

5.2.6. Proline content

Leaf proline content was quantified in five plant replicates of each treatment using the spectrophotometric method described by Shabnam et al. (2016), adapted to 96-well plates in our laboratory. Approximately 15 mg of plant material were homogenized in 500 µl of 3% aqueous sulfosalicylic acid and kept for 10 min in ice. The mixture was centrifuged at 10 ºC and 16,000 g for 10 min and the supernatant was mixed with 250 µl of glacial acetic and 500

μl of ninhydrin reagent. Then the mixture was heated at 99 °C for 40 min and immediately cooled with ice. The mixture was centrifuged and an aliquot of 200 μl transferred to a 96-well plate where the absorbance was measured at 513 nm in a FLUOStar Omega plate reader (BMG Labtech, Germany). L−proline (Acrós Organics) was used as a standard for quantification.

5.2.7. Ferric Reducing Antioxidant Potential (FRAP) assay

The total antioxidant capacity was determined in leaves of five replicates of each treatment using the ferric ion reducing antioxidant power (FRAP) method (Benzie and Strain, 1996). This method is based on the reduction of the colorless [Fe(III)−,4,6-tri(2-pyridyl)-*s*-triazine)$_2$]$^{3+}$ complex, abbreviated as Fe(III)−TPTZ, to the blue-colored Fe(II)−TPTZ complex, formed by the action of electron donating antioxidants at low pH. The FRAP reagent was prepared by mixing 300 mM acetate buffer (pH= 3.6), a solution of 10 mM TPTZ in 40 mM HCl, and 20.35 mM FeCl$_3$ at a ratio of 10:1:1 (v/v/v). Five mg of each plant sample were extracted in 700 μl of 50% aqueous acetone for 30 min in an ultrasound bath at 8 ºC. The mixture was centrifuged and transferred to a 96−well plate where 8 μl of sample, 8 μl of phosphate buffer saline, and 200 μl of FRAP reagent were added to each well. The absorbance was measured at 593 nm after 30 min in a FLUOStar Omega (BMG Labtech, Germany). A standard curve was prepared using different concentrations of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). The results were expressed as μmol trolox equivalent/g dry weight.

5.2.8. Phenolic compounds content

The content of total phenolic compounds in leaf samples (five replicates of each treatment) was determined spectrophotometrically according to the Folin−Ciocalteu method (Ainsworth and Gillespie, 2007). A 100 μl aliquot of 50% aqueous acetone extract of each sample, prepared as previously described for the FRAP essay was mixed with 500 μl of Folin-Ciocalteu reagent (Scharlab Chemie S.A.). After 5 min, a volume of 400 μl of a 700 mM Na$_2$CO$_3$ solution was added. The mixture was incubated for 60 min and the absorbance at 765 nm was measured in a 96−well plate in a FLUOStar Omega (BMG Labtech, Germany). Gallic acid was used as a reference standard, and the results were expressed as μmol gallic acid equivalent/g dry weight.

5.2.9. Antioxidant enzyme determination

The antioxidant enzymes catalase (CAT), ascorbate peroxidase (APX), and dehydroascorbate reductase (DHAR) were quantified in leaves of four plant replicates of each treatment as described in Pérez-López et al. (2009). At harvest time, three leaves of tomato plants from the same branch were pooled for antioxidant enzyme quantification. Samples of fresh leaves previously stored at -80 ºC were ground in a mortar in the presence of liquid nitrogen and then kept at -80 ºC until the measurement of the antioxidant enzyme activities.

For CAT activity, 40 mg of the ground samples were mixed with 0.5 mL of 50 mM Tris-HCl (pH= 7.8), 0.1 mM EDTA, 0.2% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mM dithiothreitol and beaten with glass beads for 1 min. The homogenates were filtered through a layer of muslin and gel-filtered over MicroSpin G25 columns (Amersham Biosciences, Sweden) equilibrated with 50 mM Tris$-$HCl (pH= 7.8), 0.1 mM EDTA and 0.2% (v/v) Triton X-100. CAT activity was measured spectrophotometrically by monitoring the disappearance of H_2O_2 at 240 nm in a reaction mixture of a final volume of 300 µl containing 50 mM potassium phosphate buffer (pH= 7.0), 25 mM H_2O_2 and 5 µl of the filtered supernatant.

The homogenizing medium for DHAR activity consisted of 50 mM potassium phosphate (pH= 7.8), 0.1 mM EDTA, 0.2% (v/v) Triton X-100, 2 mM AsA, 5 mM cysteine, 0.1 mM PMSF and 1% (w/v) poly(vinylpolypyrrolidone). An amount of 40 mg of ground samples were incubated with 0.5 ml the homogenizing buffer for 10 min at $6-8$ ºC, filtered through a layer of muslin and centrifuged at 16100 *g* for 15 min. DHAR activity was determined by monitoring AsA formation via dehydroascorbate (DHA) reduction at 265 nm. Briefly, the final volume of the assay mixture was 300 µl, and contained 2.5 mM glutathione (GSH), 0.1 mM EDTA, 50 mM potassium phosphate (pH= 6.6) and 10 µl of supernatant. The reaction was initiated by adding 10 µl of 0.2 mM DHA to the reaction mixture. The reaction rate was corrected for the non-enzymatic reduction of DHA by GSH.

For the APX activity, the ground samples were homogenized as described in the previous paragraph. APX activity was analyzed by measuring the oxidation of AsA at 290 nm. Briefly, a volume of 290 µl of reaction mixture containing 0.8 mM AsA and 50 mM HEPES (pH= 7.6) was mixed with 10 µl of the supernatant. The oxidation rate of AsA measured as the decline in absorbance at 290 nm was estimated between $1-6$ min after starting the reaction with the addition of H_2O_2 at a final concentration of 1.2 mM. Corrections were made for the non-

enzymatic oxidation of ascorbate by H_2O_2 and for the oxidation of ascorbate in the absence of H_2O_2.

The measurement of the CAT, APX and DHAR activities were carried out 25 ºC and the protein content in the supernatant was measured according to the Bradford method.

5.2.10. Statistical analysis

For each parameter, the effect of treatments (uninoculated control, *Diaporthe*, DS, *Diaporthe* + DS) was analyzed by means of one-way ANOVA and differences among their means were evaluated by a Tukey's test ($p < 0.05$). All statistical analyses were made with SigmaPlot v.14.

5.3. RESULTS

5.3.1. Effect of *Diaporthe* and water regime on plant growth

Drought stress had a strong and visible effect on tomato plants, resulting in a significant decrease in plant development (Figure 5.1). This harmful effect was reduced in plants inoculated with *Diaporthe*. In fact, *Diaporthe* reduced susceptibility to drought stress, resulting in a significant increase in several growth parameters when compared to uninoculated plants: 28% increase in shoot length, 80% in shoot biomass and 27% in root biomass (Figure 5.2A,C).

Figure 5.1 – Six-week-old tomato plants uninoculated or inoculated with *Diaporthe* strain EB4, with two different water treatments (normal watering or drought stress).
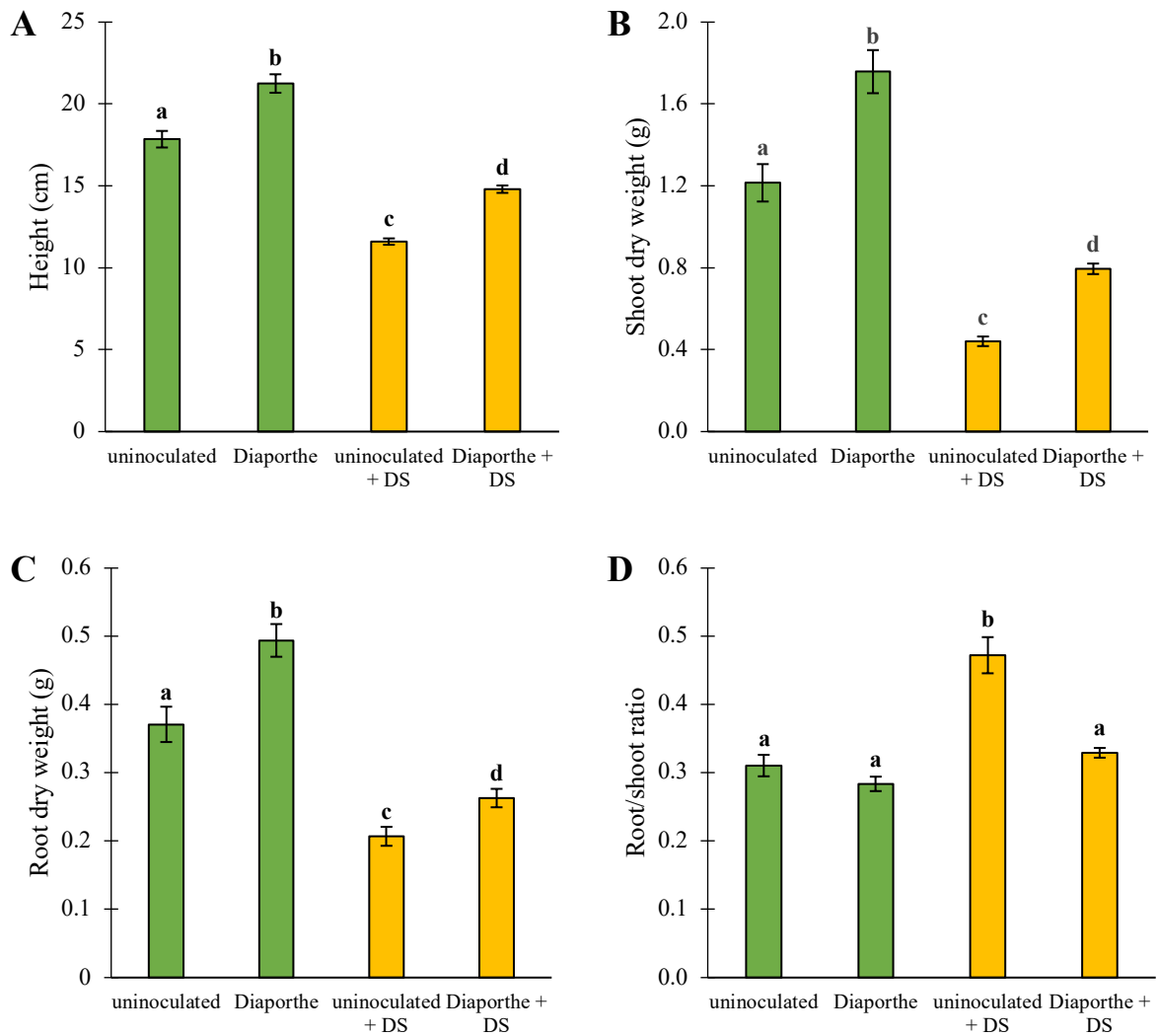
Figure 5.2 – Shoot length **(A),** shoot **(B)** and root biomass **(C),** and root/shoot ratio **(D)** of tomato plants uninoculated or inoculated with *Diaporthe* EB4 under normal water regime and drought stress (DS). For each parameter, different letters indicate significantly different means. Values are means ± SE (n=10).

Concerning to root/shoot ratio, the shoot of uninoculated plants was more sensitive to drought stress than roots resulting in a significant increase in the ratio (*p*< 0.001); in turn, plants inoculated with *Diaporthe* did not suffer any change (Figure 5.2D).

Under the normal watering treatment, plants inoculated with *Diaporthe* also showed greater shoot length, and shoot and root dry biomass than the uninoculated plants (*p*< 0.001): 19% increase in shoot length, 45% in shoot biomass and 33% in root biomass (Figure 5.2A,C).

5.3.2. Effect of *Diaporthe* and water regime on photosynthetic efficiency

Diaporthe had a positive effect on chlorophyll content, causing a significant increase when compared to uninoculated plants under normal watering and drought stress (*p*< 0.001).

Interestingly, plants with *Diaporthe* under drought stress showed the highest chlorophyll content (Figure 5.3A).
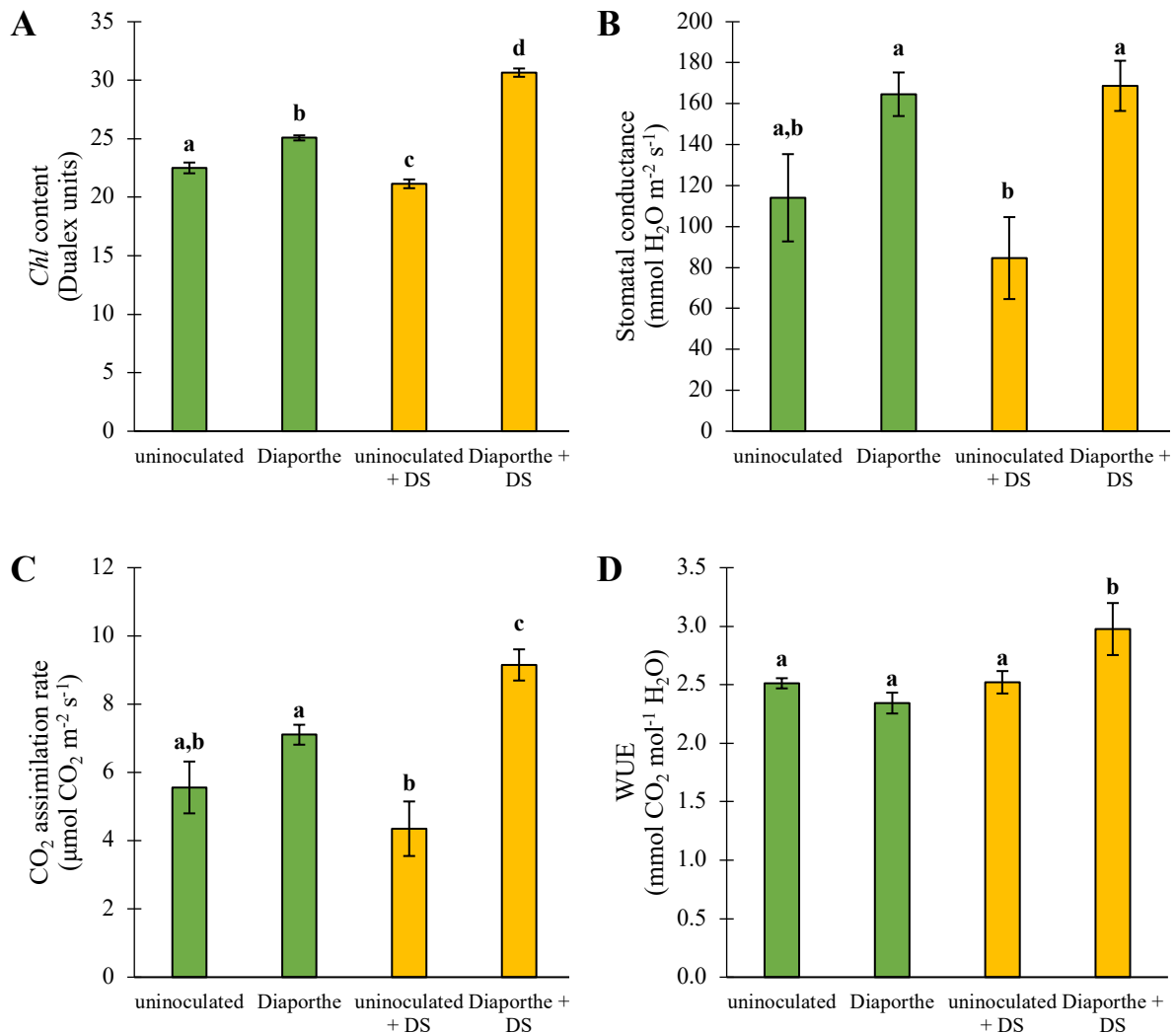
Figure 5.3 - Chlorophyll content **(A),** stomatal conductance **(B),** CO_2 assimilation rate **(C)** and water use efficiency (WUE) **(D)** of tomato plants uninoculated or inoculated with *Diaporthe* EB4, under normal water regime and drought stress (DS). For each parameter, different letters indicate significantly different means. Values are means ± SE (n=5).

The inoculation with *Diaporthe* had a significant effect on the stomatal conductance under drought stress ($p < 0.001$) (Figure 5.3B). The CO_2 assimilation rate was greater in plants with *Diaporthe*, showing a significant increase under drought stress ($p < 0.001$) (Figure 5.3C). In parallel to this, the highest WUE (water use efficiency) was detected in *Diaporthe*-plants under drought stress (Figure 5.3D).

5.3.3. Effect of *Diaporthe* and water regime on the antioxidant capacity and phenolic compounds content

The antioxidant capacity decreased significantly in plants with *Diaporthe* under drought stress ($p< 0.001$) (Figure 5.4A). Differences in total phenolic compounds between treatments were not statistically significant ($p> 0.05$) (Figure 5.4B).
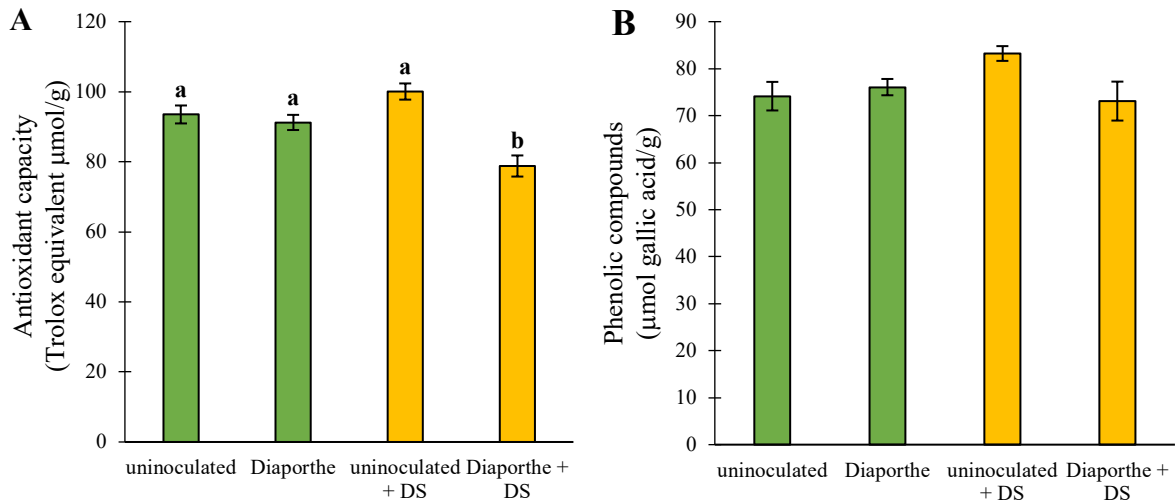
Figure 5.4 – Antioxidant capacity **(A)** and total phenolic compounds content **(B)** of tomato plants uninoculated or inoculated with *Diaporthe* EB4, under normal water regime and drought stress (DS). For each parameter, different letters indicate significantly different means. Values are means ± SE (n=5).

### 5.3.4. Effect of *Diaporthe* and water regime on the activity of antioxidant enzymes

Drought stress had a marked effect on the activity of CAT ($p= 0.002$), which was highest in *Diaporthe*-inoculated plants (Figure 5.5A). Differences in CAT between inoculated and *Diaporthe*-inoculated plants under normal watering were not significant. The activity of DHAR also increased under drought stress, but the difference was only significant in plants inoculated with *Diaporthe* ($p= 0.03$) (Figure 5.5B). In contrast APX activity decreased ($p< 0.001$) under drought stress in both uninoculated and *Diaporthe* plants (Figure 5.5C).
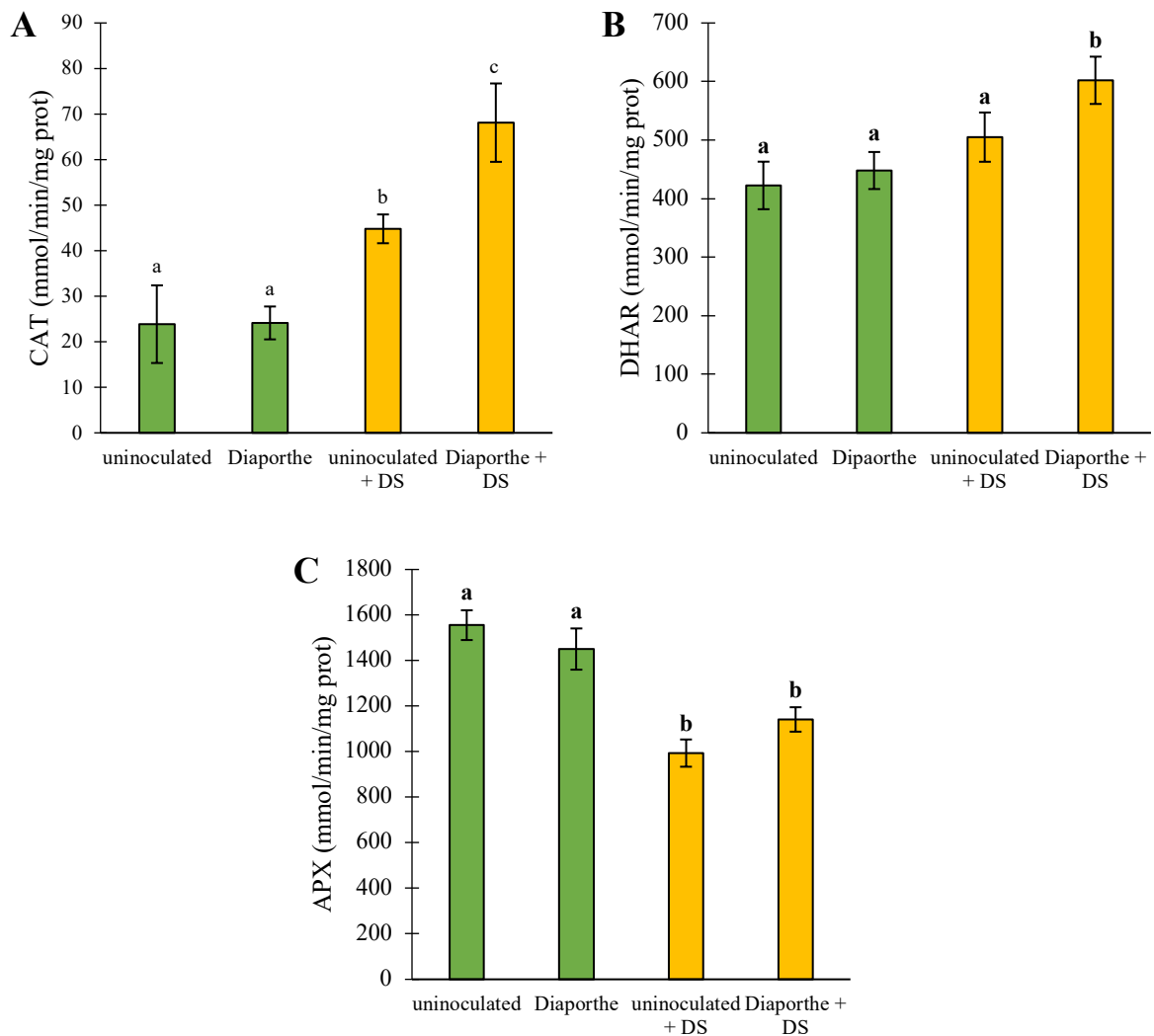
Figure 5.5 - Antioxidant enzymes catalase (CAT) **(A)**, dehydroascorbate reductase (DHAR) **(B)** and ascorbate peroxidase (APX) **(C)** of tomato plants uninoculated or inoculated with *Diaporthe* EB4, under normal water regime and drought stress (DS). For each parameter, different letters indicate significantly different means. Values are means ± SE (n=5).

5.3.5. Effect of *Diaporthe* and water regime on the proline content

The proline content increased significantly ($p < 0.001$) in plants under drought stress (Figure 5.6), and such an increase was more than twice greater in *Diaporthe*-inoculated plants than in uninoculated plants. Differences in proline between uninoculated and *Diaporthe*-plants under normal water regime were not significant.
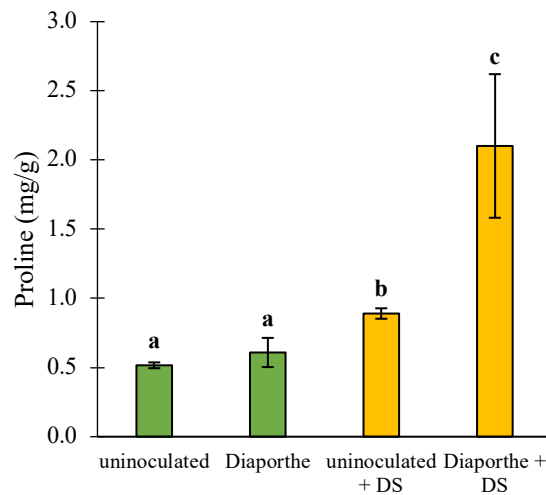
Figure 5.6 – Proline content of tomato plants uninoculated or inoculated with *Diaporthe* EB4, under normal water regime and drought stress (DS). For each parameter, different letters indicate significantly different means. Values are means ± SE (n=5).

5.3.6. Effect of *Diaporthe* and water regime on nutrient uptake

Under drought stress, the uptake of N, P and K decreased significantly in uninoculated plants (N: $p = 0.002$; P: $p = 0.003$; K: $p < 0.001$); in contrast, nutrient uptake increased (N) or was not affected (P, K) in *Diaporthe*-inoculated plants (Figure 5.7A−C). Under normal watering treatment, *Diaporthe* increased significantly the uptake of N and P (Figures 5.7A,B).
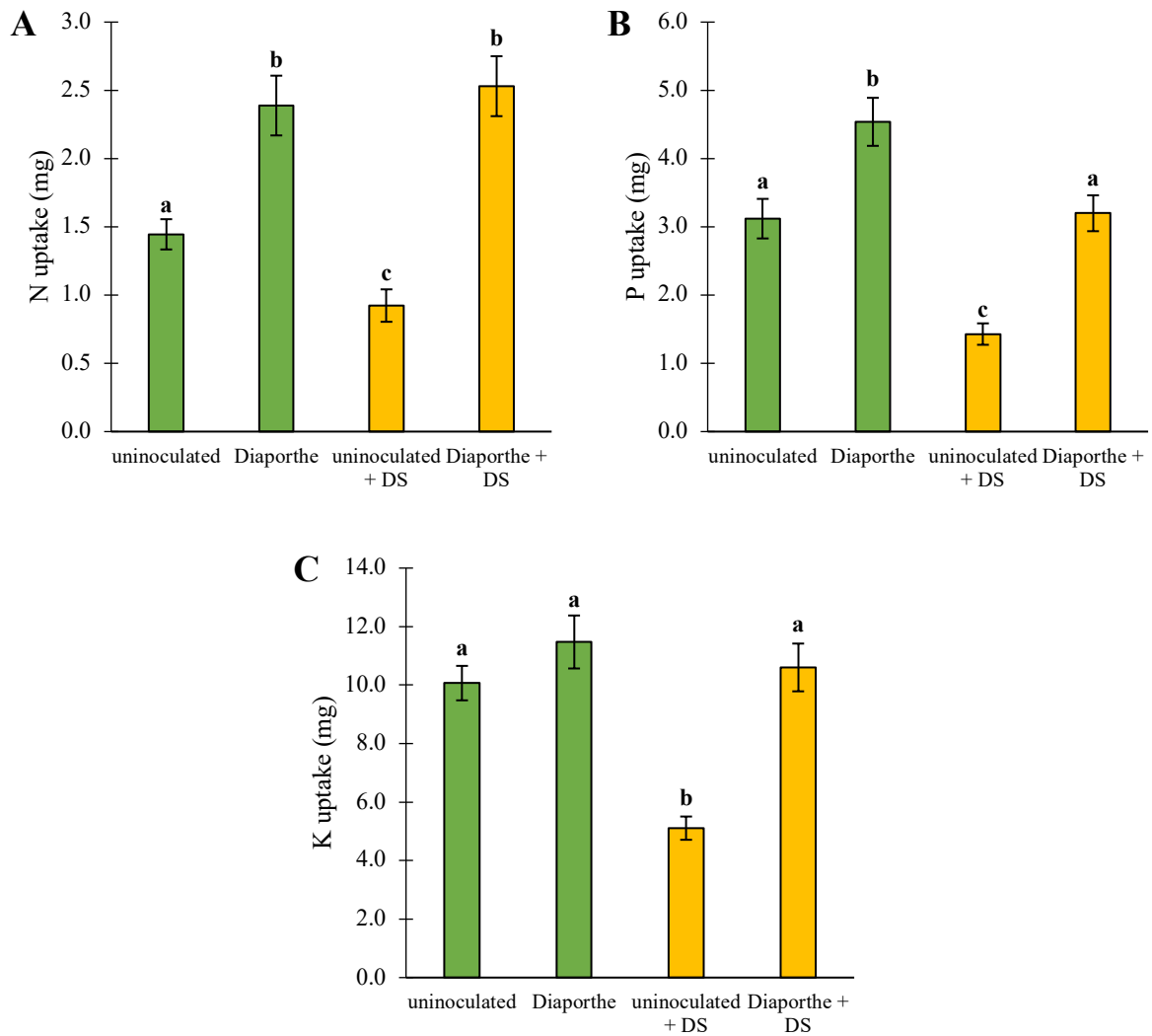
Figure 5.7 – Nitrogen **(A)**, phosphorus **(B)** and potassium **(C)** uptake by tomato plants uninoculated or inoculated with *Diaporthe* EB4, under normal water regime and drought stress (DS). For each parameter, different letters indicate significantly different means. Values are means ± SE (n=5).

5.4. DISCUSSION

In this study, we investigated the capacity of *Diaporthe* strain EB4 to promote plant growth and improve drought tolerance in tomato plants under greenhouse conditions. This strain belongs to one of the most abundant taxa of endophytes associated with the roots of the halophytic plant FRP and can enhance the response of the non-native hosts to salt stress (Chapter 2, Pereira et al., 2019; Vázquez de Aldana et al., 2019). Plant responses to drought and salinity conditions have much in common because both conditions induce osmotic stress in an early stage that leads to a decrease in growth, stomatal aperture, and deficit of nutrients (Ma et al., 2020). Therefore, plant adaptation to both stresses could be mediated by similar mechanisms involving several plant responses such as growth attenuation, accumulation of

compatible solutes as proline, increased levels of antioxidants and protective proteins, suppression of energy−consuming pathways and gene expression regulation (Bartels and Sunkar, 2005; Munns, 2011). Some fungal endophytes can mitigate the effect of both stresses, e.g., *Alternaria*, *Trichoderma, Periconia* and *Neocamarosporium* species (Azad and Kaminskyj, 2016; Moghaddam et al., 2021), although it is not clear whether the mechanisms involved in the induction of salinity stress amelioration by symbiotic microorganisms are different from those of drought stress (Ma et al., 2020).
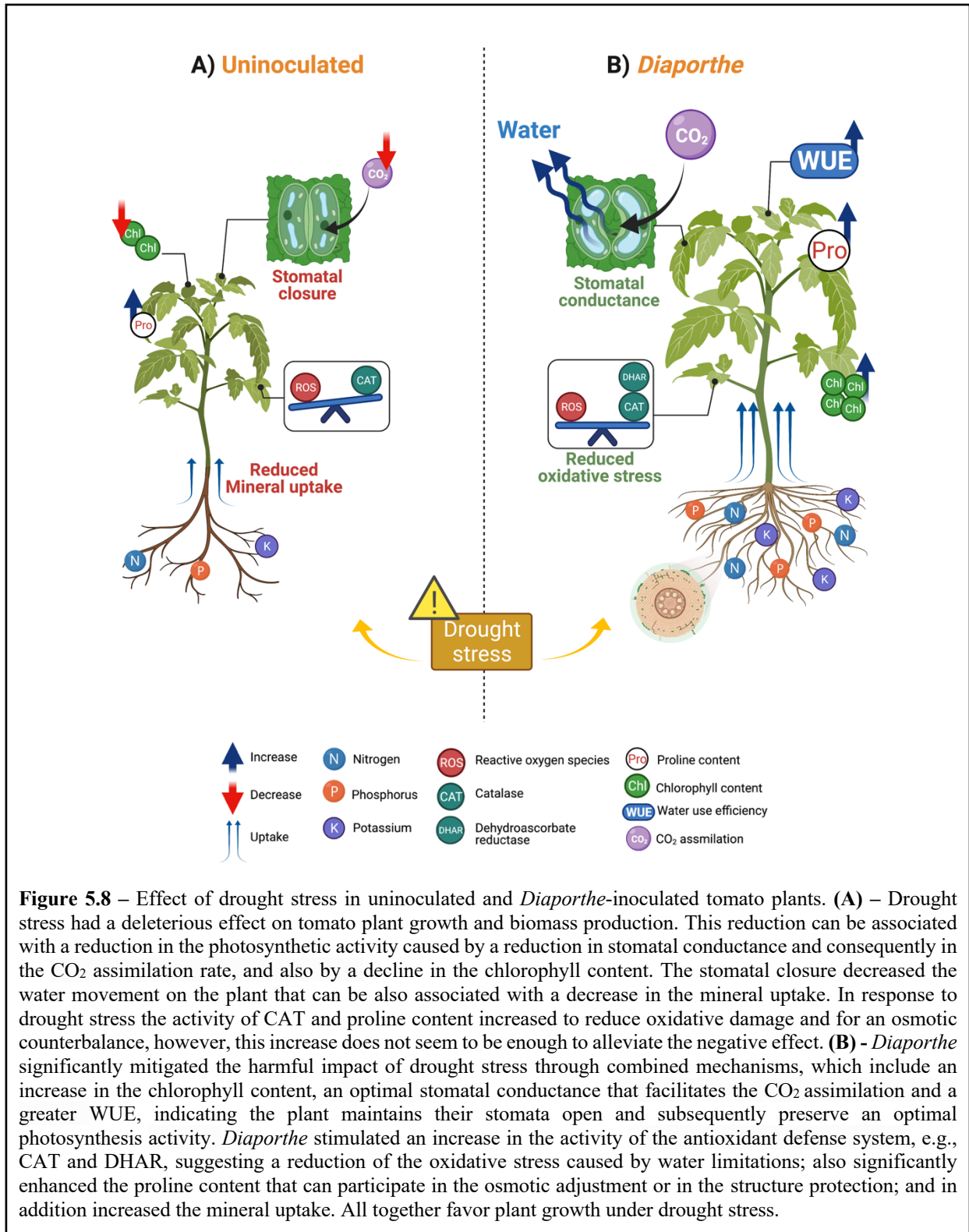
Our results showed that drought stress had a negative impact on uninoculated plants causing several morphophysiological and biochemical changes. However, plants inoculated with *Diaporthe* promoted tomato plant growth regardless of water availability, and reduced the severity of drought stress, causing a significant impact on various plant physiological parameters, as represented in the Figure 5.8. Similarly, fungal symbionts such as *Trichoderma* sp. (Azad and Kaminskyj, 2016; Khoshmanzar et al., 2020), *Ampelomyces* sp. and *Penicillium* sp. (Morsy et al., 2020), *Fusarium* sp. (Kavroulakis et al., 2018), *Talaromyces omanensis* (Halo et al., 2020), and dark septate endophytes (Valli and Muthukumar, 2018) promoted tomato plant growth and improved tolerance to drought stress. It has been reported that shoot growth is more sensitive to drought than root growth, increasing the root/shoot ratio (Hsiao and Xu, 2000; Xu et al., 2015). In fact, we found that under water limitation, uninoculated plants increased this ratio because of the high reduction in shoot growth in response to the stress; however, *Diaporthe* ameliorated the reduction in the plant growth, and thus the root/shoot ratio did not vary.

The reduction of plant growth and productivity caused by drought is frequently associated with a reduction in photosynthetic activity. The mechanism of drought stress suppression of photosynthesis is generally attributed to a stomatal limitation that avoids transpiration and a concomitant decrease in the CO_2 assimilation rate (Parkash and Singh, 2020). In the present study, water limitation caused an evident reduction in the stomatal conductance and the CO_2 assimilation rate in uninoculated plants, thereby limiting leaf photosynthesis and finally limiting tomato growth. Additionally, a decline in chlorophyll content could be considered as a symptom of oxidative stress caused by water limitation that also affects photosynthesis (Sharma et al., 2020; Kapoor et al., 2020). The reduction in the chlorophyll content under drought stress is generally correlated with damage to chloroplasts and their photosynthetic apparatus by ROS (Farooq et al., 2009; Rao and Chaitanya, 2016). In our study, a deleterious effect on photosynthesis structures of uninoculated tomato plants by ROS under water stress is also proposed, resulting in a significant decrease in the chlorophyll

content and eventually in photosynthesis inhibition. *Diaporthe* inoculation had a positive effect on the maintenance of an optimal photosynthetic activity under drought stress. Inoculated *Diaporthe* plants maintained the stomatal conductance that facilitating the entry of external CO_2 into the leaves and so the CO_2 photosynthetic assimilation, contributing to the biomass increase (Figure 5.8).

Figure 5.8 – Effect of drought stress in uninoculated and *Diaporthe*-inoculated tomato plants. **(A)** – Drought stress had a deleterious effect on tomato plant growth and biomass production. This reduction can be associated with a reduction in the photosynthetic activity caused by a reduction in stomatal conductance and consequently in the CO_2 assimilation rate, and also by a decline in the chlorophyll content. The stomatal closure decreased the water movement on the plant that can be also associated with a decrease in the mineral uptake. In response to drought stress the activity of CAT and proline content increased to reduce oxidative damage and for an osmotic counterbalance, however, this increase does not seem to be enough to alleviate the negative effect. **(B)** - *Diaporthe* significantly mitigated the harmful impact of drought stress through combined mechanisms, which include an increase in the chlorophyll content, an optimal stomatal conductance that facilitates the CO_2 assimilation and a greater WUE, indicating the plant maintains their stomata open and subsequently preserve an optimal photosynthesis activity. *Diaporthe* stimulated an increase in the activity of the antioxidant defense system, e.g., CAT and DHAR, suggesting a reduction of the oxidative stress caused by water limitations; also significantly enhanced the proline content that can participate in the osmotic adjustment or in the structure protection; and in addition increased the mineral uptake. All together favor plant growth under drought stress.

The ability of endophytes to improve photosynthesis under drought stress is often associated with the activation of biochemical pathways that reduce ROS to less harmful molecules and the induction of photosynthetic pigments (Harman and Uphoff, 2019; Harman et al. 2019). In addition, the increase in chlorophyll content due to *Diaporthe* could also be associated, at least in part, with a higher ability to assimilate N. This is in agreement with results published for *Diaporthe liquidambari* (Yang et al., 2014; 2015), where an improvement in nitrogen accumulation was observed in the presence of this endophyte. Furthermore, the chlorophyll increase can also suggest that *Diaporthe* protects the photosynthetic apparatus from possible damage caused by drought stress by the accumulation of osmoprotectant compounds. The protection of the photosynthetic structures by other symbiotic microorganisms has been observed (Saddique et al., 2018; Zhang et al., 2019). Additionally, *Diaporthe*-inoculated plants also exhibited a greater WUE under drought stress. The improvement in the ratio between photosynthesis and transpiration was responsible for a better adaptation of tomato plants to drought stress and, interestingly, the restoration of the plant growth, although it did not reach the level of those plants under the normal watering regime as a consequence of the severe reduced water availability.

Drought stress limits the efficiency of photosynthesis and causes ROS production (Muhammad et al., 2021). To reduce oxidative damage, plants have developed an antioxidant defense system, composed of enzymatic and non-enzymatic ROS scavengers (Ahmad et al., 2010). In our study, APX and CAT were affected by drought stress, although in different ways, suggesting that both enzymatic activities are important for H_2O_2 scavenging (Figure 5.8). Intriguingly, APX decreased its activity under drought stress, probably due to a lower content of ascorbate in leaf cells. This enzyme is known to be extremely labile and rapidly inactivated at low concentrations of ascorbate (Asada, 2006). Ascorbate has the ability to scavenge ROS directly or through enzymes such as APX (Pandey et al., 2017; Hasanuzzaman et al., 2019). Thus, the oxidative stress may have caused a decrease in the cellular content of ascorbate, resulting in a reduction of APX activity. This decrease is more notorious in uninoculated tomato plants under drought stress, which interestingly shows the lowest activity for DHAR. The slight, but still significant, increase in DHAR activity exhibited by *Diaporthe*-inoculated plants suggests that cellular ascorbate regeneration was better in the presence of this endophyte *Diaporthe*. The activity of CAT increased under drought stress and, particularly, enhanced by the combination of drought stress and *Diaporthe*. Despite the reduction of APX activity, all data together suggest that *Diaporthe* has the ability to improve the enzymatic antioxidant response of tomato plants under drought stress. Additionally, plants and some endophytes can

produce antioxidant compounds that may improve tolerance to oxidative stress as part of the non-enzymatic mechanism (White and Torres, 2010; Bacon and White, 2016; Varela et al., 2016), and these active antioxidants such as phenolic compounds scavenge ROS. In contrast to our expectations, *Diaporthe* seems to have a small effect or no effect on the antioxidant capacity. Previously, Vázquez de Aldana et al. (2021) detected that some endophytes, namely *Diaporthe*, could not enhance the antioxidant capacity of tritordeum but diminished it in some cases.

Osmotic adjustment through the accumulation of metabolically neutral solutes, such as proline, is an important mechanism of plant adaptation against abiotic stress, such as salinity and drought stress (Kaur and Asthir, 2015). In addition, proline interacts with protein and membranes stabilizing their structures and activities (Farooq et al., 2009; Krasensky and Jonak, 2012; Zivcak et al., 2016). As explained in Chapters 2 and 3, in response to salinity stress FRP plants synthesized and accumulated proline in leaves for an osmotic counterbalance. Crop root inoculation with endophytes like *Penicillium* sp., *Trichoderma harzianum*, DSE or *Piriformospora indica* has been reported to increase the proline content under drought and consequently the host plant tolerance (Molina-Montenegro et al., 2016; Alwhibi et al., 2017; Valli and Muthukumar, 2018; Swetha and Padmavathi, 2020). In the present study, there were no differences in proline content between *Diaporthe* and uninoculated treatments under normal watering. However, *Diaporthe*−inoculation significantly enhanced the proline content in drought-stressed tomato plants, indicating their capacity to improve drought stress adaptation, which correlated well with the phenotypic performance when exposed to this stress limitation. This increase in proline suggests that symbiotic plants have a better capacity for osmotic adjustment, structure protection and improved photosynthetic machinery, which all together favor plant growth under drought stress.

Drought stress has also a deleterious effect on the mineral nutrition of plants by reducing the mobility and uptake of individual nutrients, due to a lack of root activity, slow ion diffusion and water movement (Farooq et al., 2009; Silva et al., 2011; Kapoor et al., 2020). However, several studies have shown that plants colonized by symbiotic fungi such as arbuscular mycorrhizal fungi and root endophytes are much more efficient uptaking soil minerals (Yakti et al., 2018; Begum et al., 2019a; 2019b; White et al., 2019), which can have a positive effect on the plant adaptation to drought stress. In our study, the mineral uptake increased in plants inoculated with *Diaporthe* regardless of water treatment. *Diaporthe*-inoculated plants exhibited increased growth under both water regime conditions partly by increasing the mineral uptake. It has been reported that *Diaporthe* has the ability to increase (i)

the concentration of soluble saccharides, total free amino acids and organic acids in root exudates altering rhizospheric microbial community involved in N turnover (Yang et al., 2015), (ii) the activities of the enzymes involved in mineral cycling (Siddikee et al., 2016), and also (iii) mineral assimilation (Xie et al., 2018). Therefore, *Diaporthe* could promote the solubilization of N, P or K in the complex form in soil and facilitate their mobilization and uptake to the plant. This result suggests that the increased tolerance to drought stress mediated by *Diaporthe* could be also attributed in part to an improvement in nutrient uptake.

In agreement with previous works, in which *Diaporthe* ameliorated the growth of *Lolium perenne* and tritordeum under salinity (Vázquez de Aldana et al., 2019), this study showed that *Diaporthe* also played an important role in the adaptation of tomato to water limitations. In both experiments, *Diaporthe* increased the proline content under both stresses which are correlated with a better phenotypic performance. This osmolyte plays an important role in the adaptation to salinity and drought stress, protecting and recovering the plants more rapidly from stress. The higher proline content in symbiotic plants can be attributed to the expression increase of the gene Pyrroline-5-carboxylate synthase (P5CS), involved in proline biosynthesis, since its increase was observed in response to the inoculation of *Diaporthe* under saline conditions (Vázquez de Aldana et al., 2019). Salinity and drought stress induces osmotic stress in an early stage that leads to a decrease in growth, then an increase in proline content stimulated by *Diaporthe* seems to be a response mechanism to the adaptation. Therefore, these results suggest that this symbiotic fungus, besides being compatible with no-host natural plants from different taxonomic order, can also protect plants to both abiotic stresses at least in the early stage. These characteristics could make *Diaporthe* a promising biological agent for improving crop tolerance to abiotic stresses.

5.5. CONCLUSIONS

This study elucidates the capacity of *Diaporthe*, a symbiotic fungus adapted to a salinity environment, to promote the plant growth and biomass production and the adaptation to drought stress on tomato plants. The presence of *Diaporthe* plays a positive role in the modulation of tomato plant response to drought stress through the combination of various processes. The high stomatal conductance, which facilitates the entry of external CO_2 into the leaves and a greater water use efficiency (WUE), suggests that *Diaporthe* can ease the water transportation and can help plants to maintain their stomata open and consequently preserve an

optimal photosynthesis activity. Additionally, an increase in the chlorophyll content and in the enzymatic antioxidant response by *Diaporthe* can also be responsible for an optimal photosynthesis activity. The antioxidant defense system reduces the oxidative stress caused by ROS protecting the photosynthetic machinery. The large accumulation of proline also appears to play an important role in the response to water stress by symbiotic plants. The mechanisms by which proline decreases this stress can be divided in two general strategies, their accumulation can serve as an osmolyte in osmotic adjustment or participate directly in ROS scavenging and structure protection. A better tolerance to drought stress could also be correlated with the increase in the mineral uptake in the symbiotic plants, suggesting that the fungus has the capacity to solubilize and facilitate the mobilization of minerals to be used by the plants. Hence, an increase in the chlorophyll content could be in part associated with a better N assimilation by the *Diaporthe*-inoculated plants.

In general, these results indicates that the interaction with *Diaporthe* is an interesting strategy that induces a better response to drought stress in tomato plants. These findings reveal that plant symbiosis with beneficial root microorganisms appears to be an environmentally friendly agricultural application to mitigate the impact of climatic change on crop production.

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Chapter 6

Tomato associated with *Diaporthe* develops resistance against the pathogen *Fusarium oxysporum*

Abstract

Fungal pathogen attacks are one of the major threats to the growth and productivity of crops. Tomato, an important commercial crop around the world, is susceptible to *Fusarium oxysporum* f. sp. *lycopersici*, a pathogen that causes vascular wilt disease. Chemical treatments for the control of vascular wilt caused by *Fusarium oxysporum* have associated risks, and the interest in sustainable and environment-friendly alternatives such as resistance conferred by endophytes is increasing. In this study, we evaluated the efficacy of the *Diaporthe* fungal endophyte strain EB4 obtained from *Festuca rubra* subsp. *pruinosa* to confer resistance against *Fusarium* in tomato plants. A significant decrease in *Fusarium* wilt symptoms in plants pre-inoculated with *Diaporthe* was observed. The disease severity index was significantly reduced from 4.5 in plants inoculated only with *Fusarium* to 1.83 when plants were first pre-inoculated with *Diaporthe* and later challenged with *Fusarium*. Furthermore, pre-inoculation with *Diaporthe* diminished *Fusarium* colonization, resulting in a reduction in the vascular browning, improving the plant growth, chlorophyll content and nutrient acquisition. *Fusarium* infection caused a decrease in the expression of the jasmonic acid (JA) biosynthesis-related marker gene *AOS1*, but no significant effect on the JA marker gene *PiII* or the abscisic acid (ABA)−responsive gene *Le4* was observed.

6.1. INTRODUCTION

Diseases caused by fungal pathogens are responsible for massive losses in agricultural activities and food production around the world (Savary et al., 2012; Doehlemann et al., 2017). Chemical treatments to control fungal pathogens show some competent results, but they bring together undesirable chemical residue problems leading to environmental pollution and risks to human health (Wightwick et al., 2010). Furthermore, the resistance to many effective fungicides is widespread in some pathogen populations, compromising their control (Lucas et al., 2015). The development of pathogen-tolerant or pathogen-resistant plants is a key challenge for more economically and environmentally sustainable crop production.

Fungi have developed symbiotic associations with plants that can range from beneficial to pathogenic. For example, some *Fusarium oxysporum* strains have an endophytic lifestyle that can have a beneficial effect on plants, while *formae speciales* are pathogenic on specific plant species (Constantin et al., 2019; Edel-Hermann and Lecomte, 2019; de Lamo and Takken, 2020). The *Fusarium oxysporum* species complex has been currently grouped into 106 *formae speciales* (Edel-Hermann and Lecomte, 2019), which can cause vascular disease or root and crown rot on important crops, such as banana, cotton, soybean or tomato, producing huge agricultural and economic losses every year (Michielse and Rep, 2009; Dean et al., 2012). *Fusarium oxysporum* f. sp. *lycopersici* (Fol) is a phytopathogenic fungus that causes a destructive vascular wilt disease, limiting the production of tomato (*Solanum lycopersicum* L.) (Srinivas et al., 2019). Fol penetrates the root epidermis and spreads through the vascular tissue, colonizing and damaging xylem vessels, and eventually causing severe water stress (Singh et al., 2017).

Unlike pathogens, some endophytic fungi can colonize plant tissues and establish a beneficial symbiotic relationship, improving plant performance. This has attracted a great interest and attention in plant science and agriculture, especially in the past few years (Lugtenberg et al., 2016; Khare et al., 2018; White et al., 2019; Chitnis et al., 2020). Plant-endophyte relationships can enhance plant defense against pathogens, conferring direct mechanisms, such as where the endophytes produce antimicrobial metabolites, mycoparasitism competing by space and resources with pathogens (endophyte−pathogen interactions), or indirect mechanisms, where the endophytes stimulate systemic plant resistance mediated by phytohormone signaling, is based on initial exposure to endophytes that primes plant defense against future pathogen infection (induced resistance) (Zabalgogeazcoa, 2008; Yu et al., 2010;

Pieterse et al., 2014; Khare et al., 2018: Köhl et al., 2019). These particular characteristics of fungal endophytes can make them an alternative option to control phytopathogens.

Diaporthe is a fungal genus that comprises pathogens and endophytes of numerous plant species (Gomes et al., 2013). Some *Diaporthe* species establish asymptomatic symbiotic associations with plants growing in unhospitable environments such as *Festuca rubra* subsp. *pruinosa* (FRP)*,* a coastal grass growing found in sea cliffs (Chapter 2, Pereira et al., 2019)*.* An unidentified *Diaporthe* species was one of the dominant endophytic fungi present in roots of FRP plants and it increased the growth of *Lolium perenne* even under salinity (Chapter 2, Pereira et al., 2019).

The research on microorganisms for biological control of *Fusarium* wilt in tomato has been increasing significantly in recent years and some studies have shown that a number of microorganisms activate several mechanisms involved in plant resistance against Fol, including antibiosis, competition for nutrients, and induced systemic resistance (ISR) (Li et al., 2018; Constantin et al., 2019; 2020). ISR is a resistance mechanism in plants that is activated by a primary infection. Its mode of action depends on increasing physical and/or chemical barriers of the host plant after local induction by weak pathogens or beneficial microbes (Pieterse et al., 2014). The activation of systemic resistance against Fol depends on hormonal signaling, where salicylic acid (SA), jasmonic acid (JA), ethylene (ET) or abscisic acid (ABA) can participate to different extent (Di et al., 2016). These hormones belong to a larger signaling network that integrates environmental inputs and provides vigor against microbial attacks (Katagiri and Tsuda, 2010; Pieterse et al., 2012). While JA signaling triggers systemic resistance against necrotrophic pathogens such as Fol (Thaler et al., 2004; Glazebrook, 2005), ABA can play a regulatory function by inactivating other defense signaling pathways as do SA, JA or ET (Anderson et al., 2004; Takahashi et al., 2004; Abuqamar et al., 2006). Although it has been recognized that phytohormones can play an important role in disease development in the Fol-tomato interaction (Di et al., 2017), Constantin et al. (2019) showed that endophyte mediated resistance against Fol caused by a *Fusarium oxysporum* endophyte was independent of JA or SA signaling.

Here, we studied whether inoculation with a *Diaporthe* fungal endophyte increases resistance against *Fusarium oxysporum* f. sp. *lycopersici* in tomato plants. The response of plants to inoculation treatments was assessed in terms of their disease index, nutrient and chlorophyll content, and phytohormone related gene expression.

6.2. MATERIALS AND METHODS

6.2.1. Fungal strains

The strain *Diaporthe* EB4 was isolated from roots of a FRP plant from a natural population in the northern coast of Galicia, Spain (Chapter 2, Pereira et al., 2019). This strain was selected because it belongs to a taxon that is a component of the core microbiome of FRP roots, having an incidence in plants greater than 50% (Chapter 2, Pereira et al., 2019). *Diaporthe* mycelium used as inoculum was prepared in beet pulp medium and grown at ambient temperature for four weeks (Vázquez de Aldana et al., 2020).

Fusarium oxysporum f. sp. *lycopersici* (Fol) strain Fol029 was kindly provided by Dr. Martijn Rep (Molecular Plant Pathology; SILS, University of Amsterdam). In order to produce spores to be used as inoculum, this strain was cultured in minimal medium (0.17% yeast nitrogen base without amino acids and $(NH_4)_2SO_4$, 3% sucrose, 100 mM KNO_3) at 25 ºC and 250 rpm for one week (Barnstead Labline MaxQ4000, USA). Cultures were filtered through Miracloth (Millipore) and diluted to yield a microconidial inoculum of 1.0×10^7 spores/ml (de Lamo et al., 2018).

6.2.2. Inoculation assay

To determine the effect of *Diaporthe* EB4 as a biocontrol agent against Fol in tomato plants, a bioassay was designed with four treatments, each one with 12 single plant replicates: uninoculated control, *Diaporthe*, Fol, and *Diaporthe*+Fol. For pre-inoculation with *Diaporthe*, seeds of tomato cv. Marmande were sown in a plastic tray containing a substrate composed of seven parts of peat and perlite (1:1, v:v) previously treated at 80 °C for 24 h, and one part of *Diaporthe* inoculum. To obtain uninoculated plants, seeds were sown in a tray containing only the peat and perlite substrate. All plants were grown in a greenhouse with day-night temperature of 25 ºC. Roots of 10-day-old seedlings (24 uninoculated and 24 *Diaporthe* inoculated) were trimmed, leaving approximately 1 cm of roots to facilitate Fol infection. Then, half of the uprooted seedlings of each treatment were dipped for 5 min in a Fol spore suspension and the other half in water (de Lamo et al., 2018). All seedlings were then individually transplanted to 2.5 l plastic pots. The *Diaporthe* EB4−uninoculated seedlings with and without Fol inoculation were potted in a mix of peat and perlite (1:1, v:v) and *Diaporthe*

EB4−inoculated seedlings with and without Fol inoculation in a mix of seven parts of peat and perlite with one part (v:v) of *Diaporthe* EB4 inoculum.

Six weeks after Fol inoculation, disease symptoms were visually assessed, and plants were harvested. On each plant, a sample of three leaves from the same branch was collected, immediately immersed in liquid nitrogen and then kept at −80 ºC until use for gene expression analysis. The remaining plant material was separated into leaves, stems and roots and lyophilized. The lyophilized samples were used for measuring dry weight and chemical analysis.

6.2.3. Evaluation of *Fusarium* wilt disease symptoms

At harvest time, disease severity of the 12 plants of each treatment was estimated by means of plant biomass (shoot and root dry weight), height and the disease index (DI) as described by Gawehns et al. (2014), but adding an extra score value of 5. Thus, the DI was visually scored on a scale from 0 to 5 as follows: DI=0 no symptoms (*i.e.*, no wilt, dwarfing or brown vessels); DI=1 one or two brown vessels with no effect on plant development; DI=2 lowest fully developed leaves show chlorosis; DI=3 all fully developed leaves show chlorosis; DI=4 all leaves show chlorosis, including the rosette of newly developed leaves; and DI=5 plants are dead or very small and wilted.

To detect the presence of Fol in the vascular system of the plants, 0.5 cm thick stem pieces were sectioned at the crown and cotyledon levels, surface−sterilized, and placed on 9 cm plates containing potato dextrose agar (PDA) medium (de Lamo et al., 2018). Plates were incubated in the dark at 25 ºC for 4 d allowing the fungus to grow out from the stem sections. The Fol outgrowth was assessed at crown and cotyledon levels through morphological identification of Fol and the percentage of fungus-infected stem pieces was recorded in each plate.

6.2.4. Chlorophyll content

The chlorophyll content was determined in leaves of six-week-old tomato plants, 24 h before harvesting, by using a leaf−clip sensor (Dualex Force, Orsay, France) in six replicate plants per treatment. In each plant, three leaves of the third branch from the top were selected, and the leaf chlorophyll average was obtained from three measurements of the central position of each leaf. Chlorophyll analysis of Fol−uninoculated plants was carried out on six randomly

selected replicates, but in those Fol−inoculated it was determined only in live plants with leaves large enough for an optimal measurement of the chlorophyll content with the leaf −clip sensor.

6.2.5. Analysis of nitrogen, phosphorus, potassium, calcium, iron and manganese content

Six replicates of each treatment were analyzed for the N, P, K, Ca, Fe and Mn content. For the analysis, all aerial parts of each plant were pooled in a single sample, and dead or excessively small plants were excluded from the analysis. The P, K, Ca, Fe and Mn content was determined by inductively coupled plasma atomic emission spectroscopy (ICP−OES) in a Varian 720-ES spectrometer. Previously, freeze-dried and ground samples were calcined at 450 ºC for 8 h, and ashes were dissolved in $HCl:HNO_3:H_2O$ (1:1:8). The N content was analyzed by the Dumas combustion method in a C-N analyzer (Leco CHN−628).

6.2.6. RNA extraction and gene expression analysis

To determine the potential involvement of jasmonic (JA) and abscisic acid (ABA) in the response against Fol observed in tomato plants pre-inoculated with *Diaporthe,* the expression level of several gene markers was analyzed. Allene oxide synthase 1 (*AOS1*) is a gene involved in the biosynthesis of JA (López-Raez et al., 2010). The expression of the proteinase inhibitor II (*PiII*) gene is regulated by JA and promotes endogenous defense responses against pathogens (Uppalapati et al., 2005). The gene encoding for late-embryogenesis protein 4 (*Le4*) is involved in ABA signaling and cell protection against stress (López-Raez et al., 2010). The elongation factor 1α (*EF1α*), a constitutive expression gene, was used to normalize the gene expression level of the marker genes related to hormonal pathways (Rotenberg et al., 2006).

At harvest time, three leaves of tomato plants from the same branch were pooled for RNA extraction. Total RNA was extracted as described by Oñate-Sánchez and Vicente-Carbajosa (2008). The quality and quantity of RNA were checked using a Nanodrop ND-1000 spectrophotometer. First-strand cDNA was synthesized from 1 μg of purified total RNA using RevertAid H Minus Reverse Transcriptase (ThermoFisher Scientific) according to the manufacturer's instructions.

Gene expression was analyzed by real time qRT−PCR using TB Green Premix Ex Taq (Takara), and the gene-specific primers described in Table 6.1 in a 7900HT Fast Real-Time PCR System (Applied Biosystems).

Table 6.1 – Primers used in real-time qRT−PCR analysis of tomato defense-related gene expression. The genes monitored are markers for the jasmonic acid (JA) and abscisic acid (ABA) signaling pathways. *EF1α* was used as a housekeeping gene (HK) for normalization.

Gene	Pathway	Primer sequence (5'-3')	Reference
Allene oxide synthase 1 (*AOS1*)	JA	CACCTGTTAAACAAGCGAAAC GACCTGGTGGCATGTTCGT	López-Raéz et al. (2010)
Proteinase inhibitor II (*PiII*)	JA	GAAAATCGTTAATTTATCCCAC ACATACAAACTTTCCATCTTTA	Uppalapati et al. (2005)
Late-embryogenesis protein 4 (*Le4*)	ABA	ACTCAAGGCATGGGTACTGG CCTTCTTTCTCCTCCCACCT	López-Raéz et al. (2010)
Elongation factor 1α (*EF1α*)	HK	GATTGGTGGTATTGGAACTGTC AGCTTCGTGGTGCATCTC	Rotenberg et al. (2006)

The real time qRT−PCR conditions were 30 s at 95 ºC followed by 40 cycles of 95 ºC for 5 s, 60 ºC for 34 s and 95 ºC for 15 s, and a single step of 60 ºC for 1 min and 95 ºC for 15 s. Relative quantification of specific mRNA levels was made using the comparative method of Livak and Schmittgen (2001).

6.2.7. Statistical analyses

For each parameter, the effect of treatment (uninoculated control, *Diaporthe*, Fol, *Diaporthe*+Fol) was analyzed by means of one-way ANOVA. Then, Tukey's test ($p< 0.05$) was used to evaluate differences among means. All statistical analyses were made with SigmaPlot v.14.

6.3. RESULTS

### 6.3.1. Effect of *Diaporthe* and Fol on plant growth

Fol inoculation had a strong visible effect on tomato plants, causing a severe reduction in growth when compared to the uninoculated controls (Figure 6.1). This detrimental effect was partly counterbalanced when plants were pre-inoculated with *Diaporthe.* In fact, *Diaporthe* pre-inoculation reduced susceptibility to *Fusarium* wilt disease, resulting in a significant increase in shoot length ($p< 0.001$) (Figure 6.2A) and shoot ($p< 0.001$) and root ($p< 0.001$) dry weight production (Figures 6.2C,D) with respect to Fol plants. However, the *Diaporthe* + Fol plants did not reach the growth and development level of uninoculated plants, although a few

Diaporthe+Fol plants had no visible Fol symptoms, and showed a plant height greater than that of *Diaporthe* inoculated plants.

When tomato plants were simply pre-inoculated with *Diaporthe*, they showed a significantly smaller shoot length, and shoot and root dry biomass than the uninoculated controls (Figures 6.2A,C,D). However, when 10−day−old seedlings were uprooted for inoculation, it was observed that those *Diaporthe*−inoculated had a root system more developed than the uninoculated plants (Figure 6.3). Additonally, those plants exhibited a higher leaf/stem biomass ratio and water content than the uninoculated (Figures 6.2B,E).

Figure 6.1 – Six-week-old tomato plants uninoculated, inoculated with *Diaporthe* sp. EB4, infected with *Fusarium oxysporum* f. sp. l*ycopersici* (Fol), and inoculated with *Diaporthe* sp. EB4 before infected with Fol.
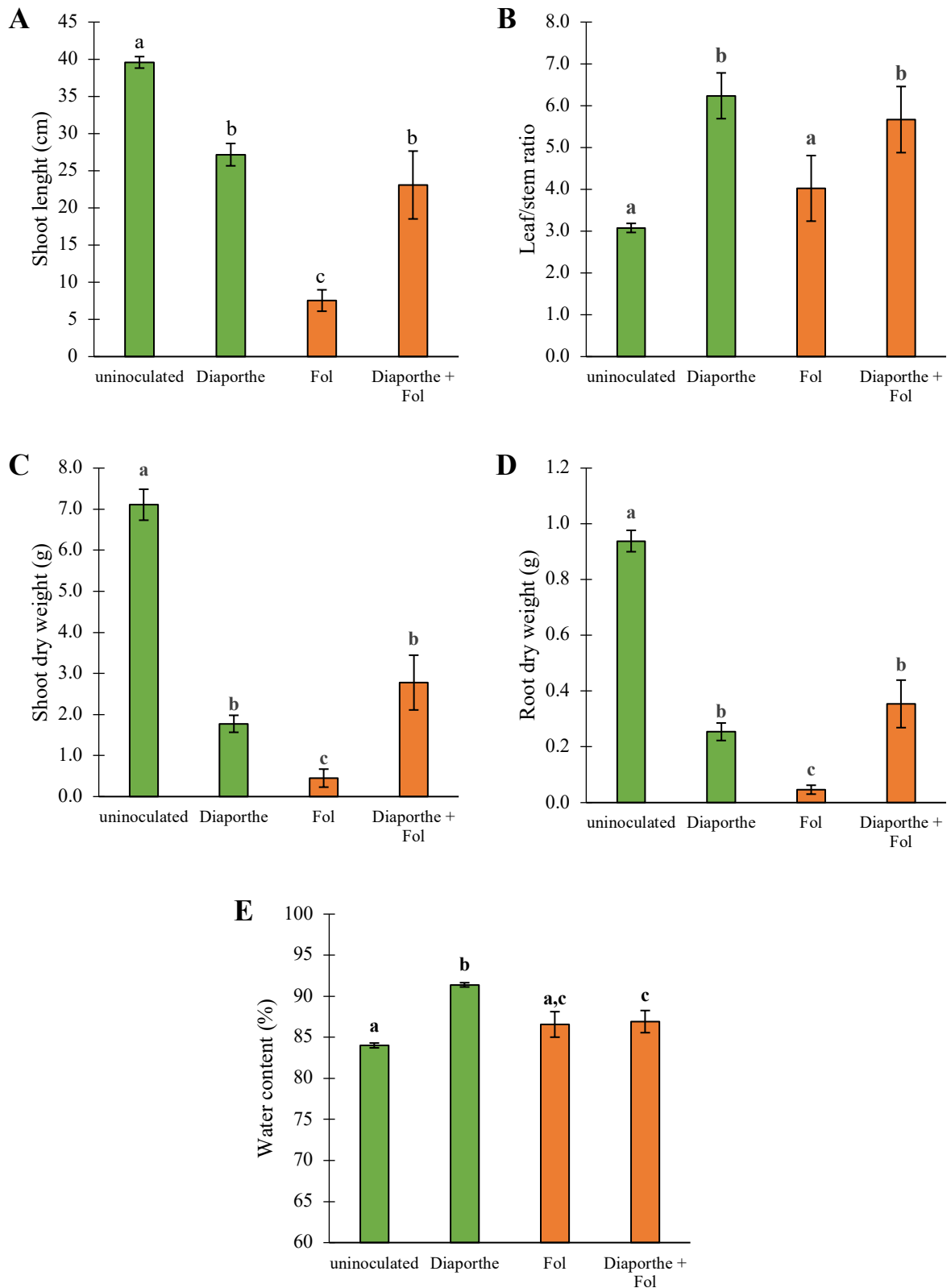
Figure 6.2 – Shoot length **(A)**, leaf/stem ratio **(B)**, shoot **(C)** and root biomass **(D)** and water content **(E)** of six-week-old tomato plants uninoculated, inoculated with *Diaporthe* sp. EB4, infected with *Fusarium oxysporum* f. sp. l*ycopersici* (Fol), and inoculated first with *Diaporthe* sp. EB4 and later with Fol. Different letters indicate significantly different means ($p < 0.05$). Values are means ± SE (n=12).
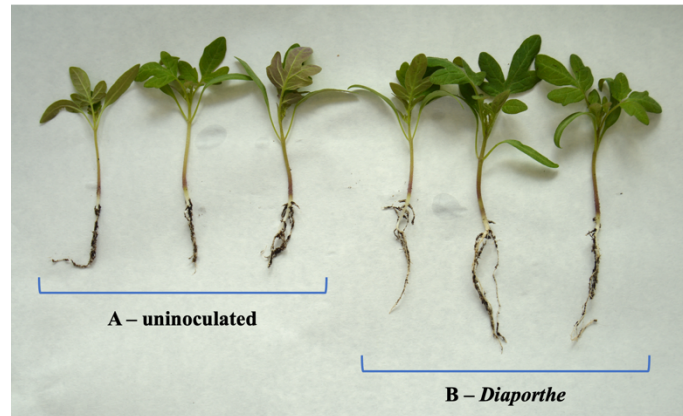
Figure 6.3 – 10-day-old seedlings used for *Fusarium oxysporum* f. sp. *lycopersici* inoculation shown before its root was trimmed. (**A**) seedlings germinated in substrate without inoculum; (**B**) seedlings germinated in subtract with *Diaporthe* inoculum. The seedlings germinated in *Diaporthe* showed a better development than the uninoculated.

6.3.2. Effect of *Diaporthe* on Fol disease severity

The Disease Index (DI) of plants infected with Fol was significantly reduced ($p < 0.001$) in plants pre-inoculated with *Diaporthe* (Figure 6.4). Plants with Fol showed severe symptoms: 50% of them showed chlorosis in all leaves (DI= 4) and the remaining ones were either dead or small and wilted (DI= 5). However, when plants were pre-inoculated with *Diaporthe*, the Fol disease symptoms diminished: 42% of the plants were symptomless (DI= 0), 25% showed mild symptoms (DI= 1−3), and 33% showed severe Fol wilt symptoms (DI= 4−5). Summarizing, the mean DI score in Fol-infected plants was 4.5, but it decreased to 1.8 in *Diaporthe* + Fol.
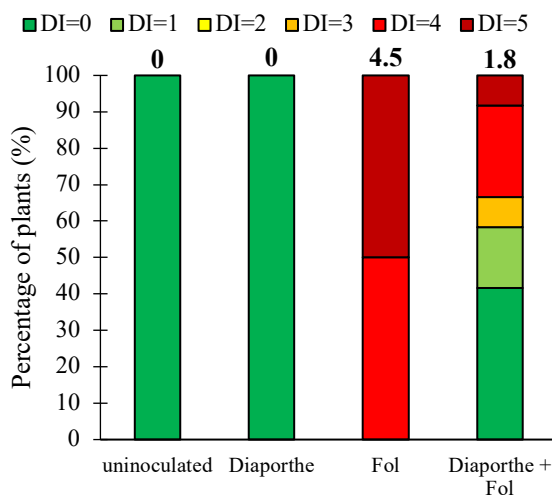
Figure 6.4 – Quantification of the Disease Index (DI) in tomato plants after inoculation with Fol and *Diaporthe*. Percentage of plants on each DI category: DI=0 no symptoms; DI=1 one or two brown vessels and development not affected; DI=2 lowest fully developed leaves show chlorosis; DI=3 all fully developed leaves show chlorosis; DI=4 all leaves show chlorosis, including the rosette of newly developed leaves; DI=5 dead or very small and wilted. The mean DI score of each treatment is indicated above each column.

In order to determine to what extent Fol colonization was inhibited by *Diaporthe*, surface-sterilized stem sections at the crown and cotyledon levels were placed on PDA medium. Stem pieces from uninoculated and *Diaporthe*−inoculated plants did not show any fungal outgrowth at the crown or cotyledon level (Figure 6.5). Fol outgrowth was observed in all stem sections from plants inoculated with Fol at cotyledon level and in 92% at the crown level. In contrast, 58% of the *Diaporthe*+Fol plants showed Fol infection at the cotyledon level and only 35% at the crown level. In 42% of plants, no infection of any stem piece was observed (Figure 6.4).
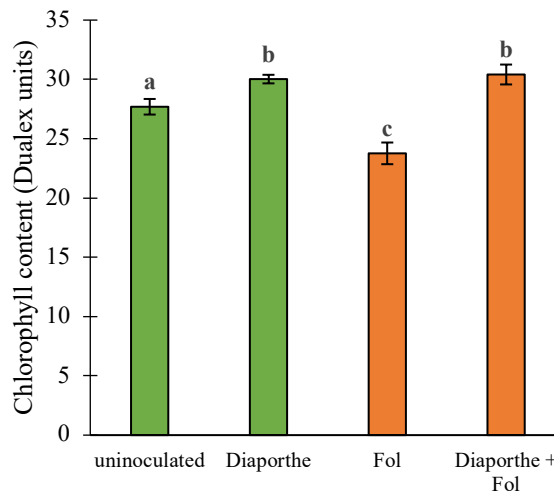

Figure 6.5 – Isolation of *Fusarium oxysporum* f. sp. *lycopersici* (Fol) from stem pieces at the cotyledon level of tomato plants: **(A)** Stem pieces from different plants with several levels of Fol infection, from asymptomatic (without xylem vessel infection by Fol) to severe Fol wilt symptoms (with xylem vessel infection by Fol). **(B)** Stem pieces from a plant of each treatment incubated for 4 d on minimal medium, uninoculated and *Diaporthe*-inoculated plants without fungal outgrowth, Fol inoculated plant with fungal outgrowth in all pieces and different levels of fungal outgrowth in *Diaporthe* + Fol plants, without fungal outgrowth and fungal outgrowth in some pieces.

### 6.3.3. Effect of *Diaporthe* and Fol on chlorophyll content

The chlorophyll content decreased significantly ($p< 0.001$) in leaves of plants inoculated with Fol (Figure 6.6). However, *Diaporthe* inoculation had a positive effect on chlorophyll content, causing a significant increase when compared to control plants. This could be observed visually in the more intense green color of the leaves of plants pre-inoculated with *Diaporthe* (Figure 6.1). It is of interest that Fol infection did not change the chlorophyll content of plants pre-inoculated with *Diaporthe*.

Figure 6.6 – Effect of the inoculation with *Diaporthe* and/or *Fusarium oxysporum* f. sp. *lycopersici* (Fol) on the chlorophyll content of tomato plants. Different letters indicate significantly different means ($p< 0.05$). Values are means ± SE (n=6).

6.3.4. Effect of *Diaporthe* and Fol on mineral content

The content of N, P, K, Ca, and Fe significantly increased with all inoculation treatments ($p< 0.001$ for all elements) (Figure 6.7). The content of N, P, and K and Fe was greater in plants inoculated with *Diaporthe* than in *Diaporthe*+Fol. The highest Ca content was detected in both Fol and *Diaporthe*+Fol plants. The Fol plants showed the highest K content and also the higher values for P, Ca and Fe. Mn only increased with Fol infection.
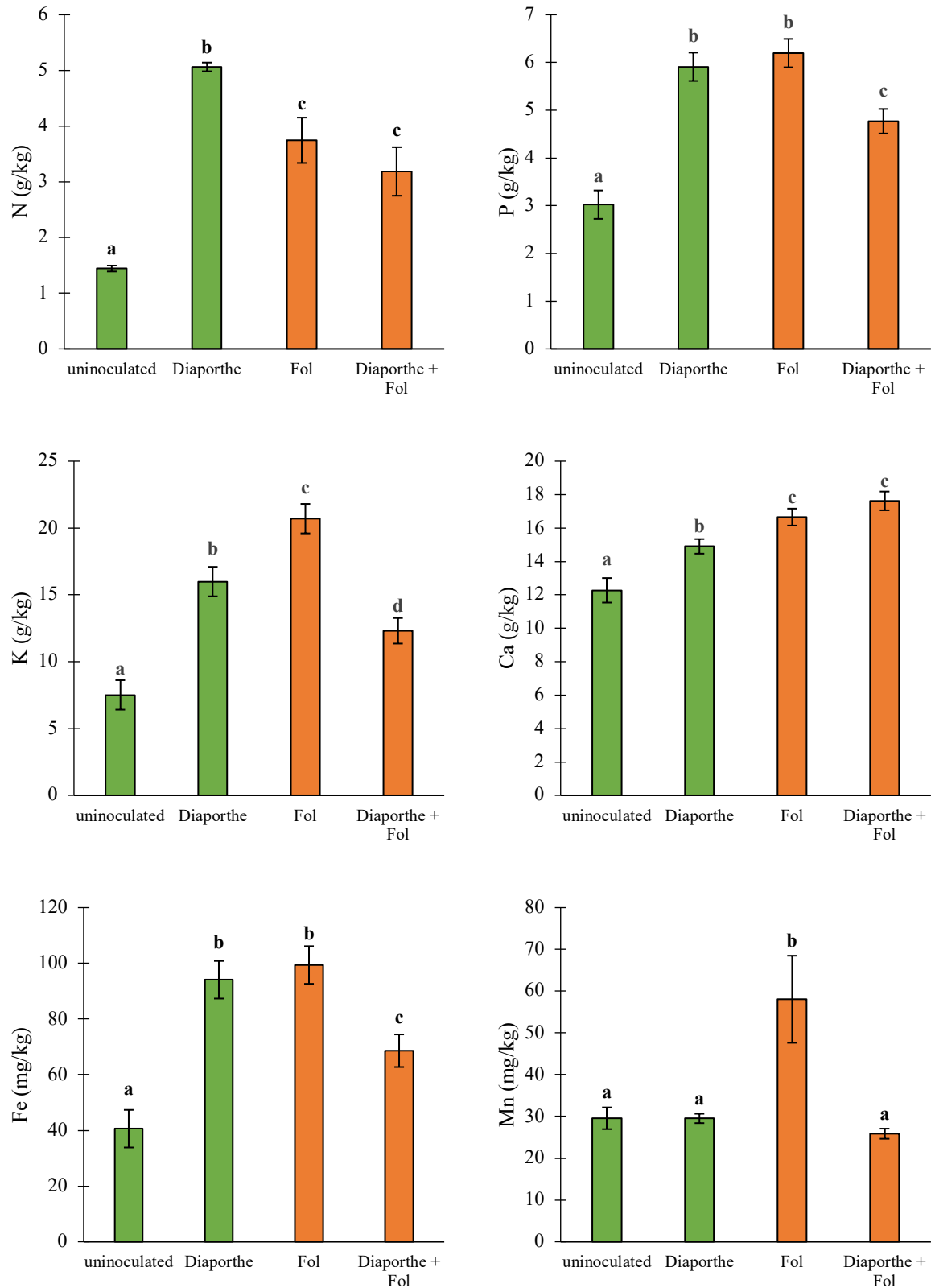
Figure 6.7 – Effect of *Diaporthe* and *Fusarium oxysporum* f. sp. *lycopersici* (Fol) on nitrogen, phosphorus, potassium, iron and manganese concentration of tomato plants. Different letters indicate significantly different means ($p < 0.05$). Values are means ± SE (n= 6).

6.3.5. Involvement of JA and ABA signaling pathways in the plant defense against *Fusarium oxysporum*

To determine whether phytohormone signaling plays a key role in the beneficial effect observed by *Diaporthe* against Fol, the expression level of several JA and ABA gene markers was analyzed by real time qRT−PCR. The infection with the pathogenic Fol caused a significant ($p= 0.002$) decrease in the expression level of the JA biosynthesis-related gene marker *AOS1* when compared to the uninoculated plants (Figure 6.8A). In contrast to the decrease in the *AOS1* expression in Fol plants, the inoculation with *Diaporthe* caused a significant ($p< 0.001$) increase in the expression level of the JA responsive gene marker *PiII* when compared to the rest of treatments (Figure 6.8B).

To elucidate the role of other hormonal signaling pathways in plant fitness recovery, the expression level of genes involved in the ABA signaling pathway was also analyzed. The expression level of *Le4* did not show significant ($p> 0.05$) differences among treatments (Figure 6.8C).
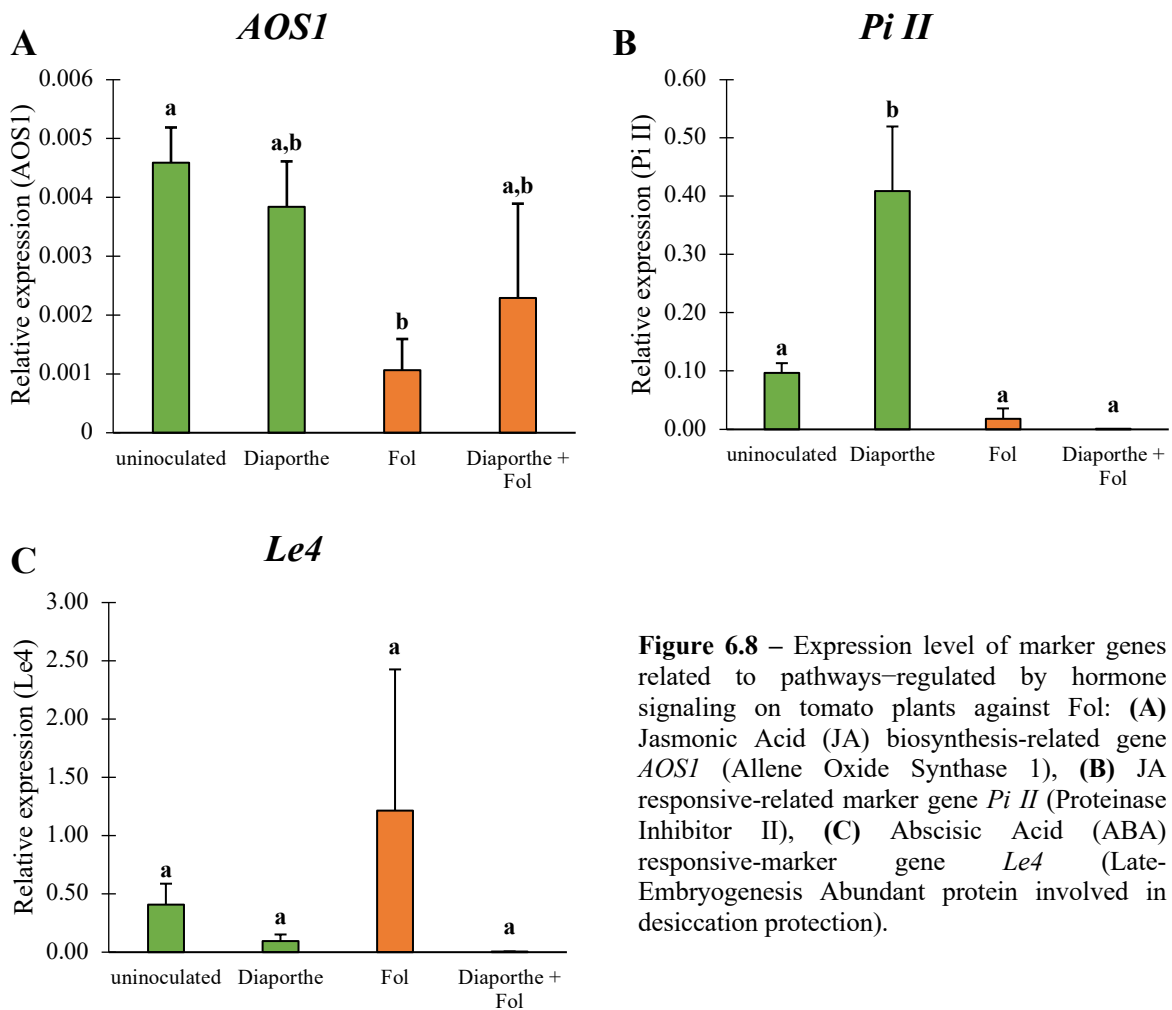
Figure 6.8 – Expression level of marker genes related to pathways−regulated by hormone signaling on tomato plants against Fol: **(A)** Jasmonic Acid (JA) biosynthesis-related gene *AOS1* (Allene Oxide Synthase 1), **(B)** JA responsive-related marker gene *Pi II* (Proteinase Inhibitor II), **(C)** Abscisic Acid (ABA) responsive-marker gene *Le4* (Late-Embryogenesis Abundant protein involved in desiccation protection).

6.4. DISCUSSION

In this study, we found that *Diaporthe* strain EB4 can protect plants against the phytopathogenic *Fusarium oxysporum* f. sp. *lycopersici* (Fol). As expected, tomato plants inoculated with Fol showed wilt symptoms derived from vascular damage, a reduction in growth, and plant death in some cases. The preinoculation of plants with *Diaporthe* EB4 significantly reduced the disease severity by inhibiting partly or totally Fol colonization of the xylem vessels, causing on average an increase in chlorophyll, and root and shoot biomass; although 33% of the plants still showed severe Fol symptoms. Similarly, other microorganisms have been reported to mitigate *Fusarium* wilt in tomato, including non-pathogenic endophytic strains of *Fusarium oxysporum* (Constantin et al., 2019; 2020), *Trichoderma* (Sallam et al., 2019; Patel and Saraf, 2017), a mycorrhizal *Glomus* species (Song et al., 2015), and several bacteria (Ben Abdallah et al., 2016; Jangir et al., 2018).

Although *Diaporthe* did not cause any disease symptom, inoculated plants had less biomass than those uninoculated. This might be related to the technique used to inoculate Fol into the tomato seedlings by cutting their roots. In a previous study (Chapter 5), the same *Diaporthe* strain promoted the growth of tomato plants, even under drought stress conditions, but in that case the roots were not trimmed. In fact, when 10-day-old seedlings were uprooted for inoculation, it was observed that those *Diaporthe*-inoculated had a root system more developed than the uninoculated ones (Figure 6.3). Thus, the lower biomass of *Diaporthe*-inoculated plants in this experiment could be related to the root trimming, which may have affected *Diaporthe* colonization and consequently plant growth.

Inoculation with *Diaporthe* induced a higher chlorophyll content, and this effect was even visually recognizable because these leaves were greener than those in uninoculated plants. This increase in the chlorophyll content stimulated by the presence of *Diaporthe* has also been observed in Chapter 5. Similar to our results, an increase in chlorophyll mediated by endophytes has been reported for *Diaporthe* (Wężowicz et al., 2017), *Trichoderma* (Zhang et al., 2016; Herman et al., 2019), dark septate endophytes (Zhang et al., 2012), and *Piriformospora indica* (Shahabivand et al., 2017; Ghorbani et al., 2018; Li et al., 2020). The increase in the chlorophyll content caused by *Diaporthe* could be partly associated with a higher ability to assimilate N as observed in other *Diaporthe* species that increased the nitrogen supply to the plant host and significantly enhanced the chlorophyll content (Siddikee et al., 2016). This could be related to an increase in the activities of the enzymes involved in mineral

cycling (Yang et al., 2014; Siddikee et al., 2016), or with an effect in the rhizospheric microbial involved in N turnover (Yang et al., 2015) promoted by the fungus.

The soilborne fungus Fol infects plants through the roots and colonizes the xylem, causing its blockage. This blockage decreases water flow within the plant, resulting in leaf water deficit and, consequently, a reduction in transpiration and leaf photosynthetic rates (Lorenzini et al., 1997; Nogués et al., 2002). In fact, our results showed a significant reduction in the chlorophyll content of plants infected with Fol, suggesting a lower photosynthetic activity. Additionally, the xylem blockage can also affect the uptake, translocation and utilization of nutrients (Mehrotra, 2013). However, our results showed that plants infected with Fol had a significant increase in nutrient content (N, P, K, Ca, Fe and Mn). Since plant growth was significantly reduced, this nutrient content increase may be associated that that in a primordial stage of development the first leaves acted as a source of minerals and photosynthates for the later developing leaves, which acted as sink until they develop their photosynthetic apparatus. The inhibition of plant growth by Fol and subsequent arrest of new leaf development may have resulted in the nutrient accumulation observed. When plants were pre−inoculated with *Diaporthe* before Fol infection, an increase in chlorophyll and nutrient content was detected, which can be related to the suppression of the Fol disease. It has been reported that *Trichoderma* sp. and endophytic *Bacillus* bacteria augmented the chlorophyll content and improved the acquisition of nutrients of tomato and cucumber seedlings, respectively, under *Fusarium* wilt infection (Li et al., 2018; 2019; Shahzad et al., 2017).

Interestingly, the Mn content only increased in plants infected by Fol. It is known that Mn is involved in pathogen defense through lignin production, an important biochemical barrier against fungal invasion (Dordas, 2008; Orr and Nelson, 2018), and in the production of phenolic compounds that are toxic to pathogens (Graham and Webb, 1991). Thus, Mn accumulation in Fol plants might be associated with high demand due to a defense response of the plants against *Fusarium*. In plants pre-inoculated with *Diaporthe* before Fol infection the Mn content was not affected. This can be understood as a defense involving Mn against Fol was not activated by the presence of the symbiotic fungus.

Diaporthe reduced the proliferation of Fol colonization on xylem stem vessels and most plants only showed Fol infection at cotyledon level or no infection at all. Both fungi were inoculated in the roots, and this suggests that the presence of *Diaporthe* can reduce the amount of the pathogen in this part of the plant, limiting Fol spread. In fact, this reduction of pathogen colonization correlates with the reduction of disease symptoms like yellowing and leaf wilting as shown. Therefore, the resistance observed in our study implies the possibility of *Diaporthe*

limiting the propagation of Fol *via* direct mechanisms, such as by antibiosis, or competition for nutrients and niches. Similarly, Constantin et al. (2020) observed that endophytic strains of *Fusarium oxysporum* reduced the root and stem colonization of Fol in tomato plants.

The pathogenic fungus *F. oxysporum* with its different *formae speciales,* such as *lycopersici*, is considered a hemibiotrophic pathogen, which starts as a biotroph and later changes to a necrotrophic lifestyle (Michielse and Rep, 2009; Chen et al., 2014). This lifestyle change requires Fol to be able to hijack host defense signaling pathways, as the JA signaling machinery (Thatcher et al., 2009; Chen et al., 2014; Di et al., 2016). Certain endophytes are capable of eliciting induced systemic resistance (ISR) against pathogens, which is regulated by a network of interconnected signaling pathways where phytohormones such as JA play a major regulatory role (Robert-Seilaniantz et al., 2011; Antico et al., 2012; Pieterse et al., 2014). In our study Fol infection caused a decrease in the expression of the JA biosynthesis-related marker gene *AOS1*, suggesting that Fol might affect the JA pathway to suppress defense responses and infect the tomato plants. In contrast, tomato plants inoculated with *Diaporthe* + Fol, or *Diaporthe* alone showed a *AOS1* expression level similar to uninoculated plants and plants infected with Fol. However, JA was only determined six weeks after inoculation and a possible accumulation of JA in an early stage of *Diaporthe* inoculation should not be ruled out. Inoculation with symbiotic fungi caused a transient accumulation of JA in an early stage of inoculation, implying a transient role of the hormone early in host colonization (Shoresh et al., 2005; Aimé et al., 2013; Veloso et al, 2016). In fact, this JA activation by *Diaporthe* may have happened because the expression of the JA-responsive gene *PiII* is still expressed in *Diaporthe*-inoculated plants. Nevertheless, *PiII* was not induced in plants with *Diaporthe* under Fol infection. Our results suggest that ISR is not activated by *Diaporthe*, in agreement with previous studies in tomato that demonstrated that endophyte-mediated resistance against Fol was independent of the conventional defense and hormone pathways (Constantin et al., 2019; 2020; de Lamo et al., 2020). ABA has been recognized as critical regulator of Fol interaction with positive or negative impact (Anderson et al., 2004; Trusov et al., 2009; Di et al., 2016). In our study, the expression of ABA-responsive gene *Le4* did not increase significantly in plants infected with Fol, and it was absent in *Diaporthe* and *Diaporthe*+Fol plants. This suggests that ABA was not involved in Fol-mediated disease development and did not play a major role in the effect of *Diaporthe* in enhancing resistance to *Fusarium*, or a visible effect happened in an early stage of infection.

6.5. CONCLUSIONS

This research shows the potential of the symbiotic fungus *Diaporthe* to confer resistance against the pathogen *Fusarium oxysporum* f. sp. *lycopersici*. *Diaporthe* can reduce the severity of *Fusarium* wilt of tomato plants by diminishing the colonization of vessels and wilt symptoms, while increasing root and shoot biomass, chlorophyll and nutrient contents. However, it is unknown if the resistance increase against *Fusarium* is induced systemic resistance or due depends on direct mechanisms that limit *Fusarium* colonization inside roots and stems. Control of *Fusarium* wilt disease is an important challenge, therefore understanding the mechanisms involved in the suppression of this disease by the symbiotic fungus *Diaporthe* will allow to improve new techniques and strategies for better control. Additionally, the root trimming method used for *Fusarium* inoculation appears to cause a delay in the development of *Diaporthe* inoculated plants, hence in further studies alternative inoculation methods would be developed.

6.6. REFERENCES

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General conclusions

This thesis aimed to understand the role of the native mycobiota in the adaptation of plants to stressful environments. In addition to determining the incidence of *Epichloë festucae* on natural populations, the culturable endophytic mycobiota of roots of *Festuca rubra* subsp. *pruinosa* was investigated for the first time (Chapter 2). Furthermore, the properties of the mycobiota in the adaptation of *Festuca rubra* to saline conditions were investigated at physiological, transcriptomic and metabolomic levels (Chapter 3 and 4). The potential of *Festuca rubra* root endophytes for applications as plant growth promoting agents, and for the improvement of tolerance to abiotic and biotic stresses in tomato plants were also evaluated (Chapter 5 and 6).

The roots of *Festuca rubra* subsp. *pruinosa* constitute a niche where an assemblage of 135 culturable fungal species was identified. The high incidence of seven species that were present in more than 20% of the plants, and in several populations was remarkable. These species were *Fusarium oxysporum*, *Diaporthe* sp. A, *Fusarium* sp. A, *Helotiales* sp., *Drechslera* sp., *Slopeiomyces cylindrosporus*, and *Penicillium* sp. F., and they seem to be components of the core microbiome of *Festuca rubra*.

The incidence of *Epichloë festucae* was relatively high, about 66% of the *Festuca rubra* plants were infected by this fungus. However, the presence of *E. festucae* in aboveground plant parts did not have an obvious effect on the composition of the core microbiome or other root mycobiota.

Due to the high prevalence of *Epichloë* in the aboveground parts, and of some species of fungi in the roots, the effect of *E. festucae* and the root endophytes *Fusarium oxysporum* and *Periconia macrospinosa* on the performance of *Festuca rubra* subsp. *pruinosa* subjected to salinity was studied. *Festuca rubra* appeared to cope with salinity through a tissue tolerance mechanism that allowed its cells to accumulate Na^+, possibly in vacuoles, which went together with an osmotic counterbalance that occurred in the cytoplasm by means of accumulating proline and K^+. Additionally, *Epichloë festucae*, *Fusarium oxysporum* and *Periconia macrospinosa*, components of the *Festuca rubra* core microbiome, contributed with different functions, which were beneficial and complementary for plant adaptation to its natural saline habitat: (i) *Epichloë* caused a Na^+ reduction in leaves under salinity which might be associated with salinity tolerance and plant survival in the long term. (ii) *Fusarium oxysporum* promoted the growth of leaves and roots in the presence as well as in the absence of salinity, and caused a decrease in leaf Na^+ content under salinity which could improve plant adaptation. (iii) *Periconia macrospinosa* promoted the growth of leaves and roots of *Festuca rubra* plants regardless of the salinity treatment.

A set of 2304 plant genes showing differential expression in response to salinity and the symbiosis with *Epichloë* was identified, showing that salinity had a major effect on gene expression, while the presence of *Epichloë* endophyte only seemed to slightly modulate salt-related gene expression. The increased expression of genes involved in ion transport and proline biosynthesis confirmed the previously observed NaCl tissue tolerance mechanism that the plant uses to cope with salinity. Additionally, salinity caused the down-regulation of genes associated with photosynthetic light-harvesting, possibly to prevent photoinhibition of photosynthesis and subsequent oxidative damage.

The metabolomic analysis showed that *Festuca rubra* extract profiles were similar among treatments, and based on relative mass defects the majority of ions detected corresponded to terpenoids. A remarkable increase in the abundance of two compounds occurred in plants infected with *Epichloë*, regardless of the salinity regime. Both compounds were constituted by fragments of dialanine but they contained a still unidentified part.

The potential of fungal endophytes isolated from *Festuca rubra* roots for crop improvement was considered under different stress factors:

A *Diaporthe* strain ameliorated the salt stress response in the grass *Lolium perenne* and also promoted plant growth without the stress.

The presence of *Diaporthe* also played a positive role in the response of tomato plants to drought stress. *Diaporthe* significantly mitigated the harmful impact of drought stress through combined mechanisms, which included an increase in the chlorophyll content and better stomatal conductance, indicating the plant preserved an optimal photosynthesis activity. *Diaporthe* also stimulated the increase of the antioxidant defense system, suggesting a reduction of the oxidative stress caused by water limitations; significantly enhanced the proline content and increased the mineral uptake. Interestingly, the positive effect of *Diaporthe* was not only limited to drought stress, under normal watering the symbiotic plants, but also showed greater growth and biomass production. These results suggested that symbiotic *Diaporthe* strain, besides being compatible with other plant species than its natural host, could also promote tomato development and protect it against abiotic stress, at least in an early stage of growth.

Diaporthe reduced the damage caused by *Fusarium* wilt in tomato plants by diminishing the colonization of vessels, resulting in a reduction in the wilt symptoms and vascular browning that went together with an improvement of the plant growth, chlorophyll content and nutrient acquisition. However, it is currently unknown whether the resistance

promoted by *Diaporthe* against *Fusarium* was triggered by induced systemic resistance, or it depended on a direct mechanisms that limited *Fusarium* entry in roots and stems.
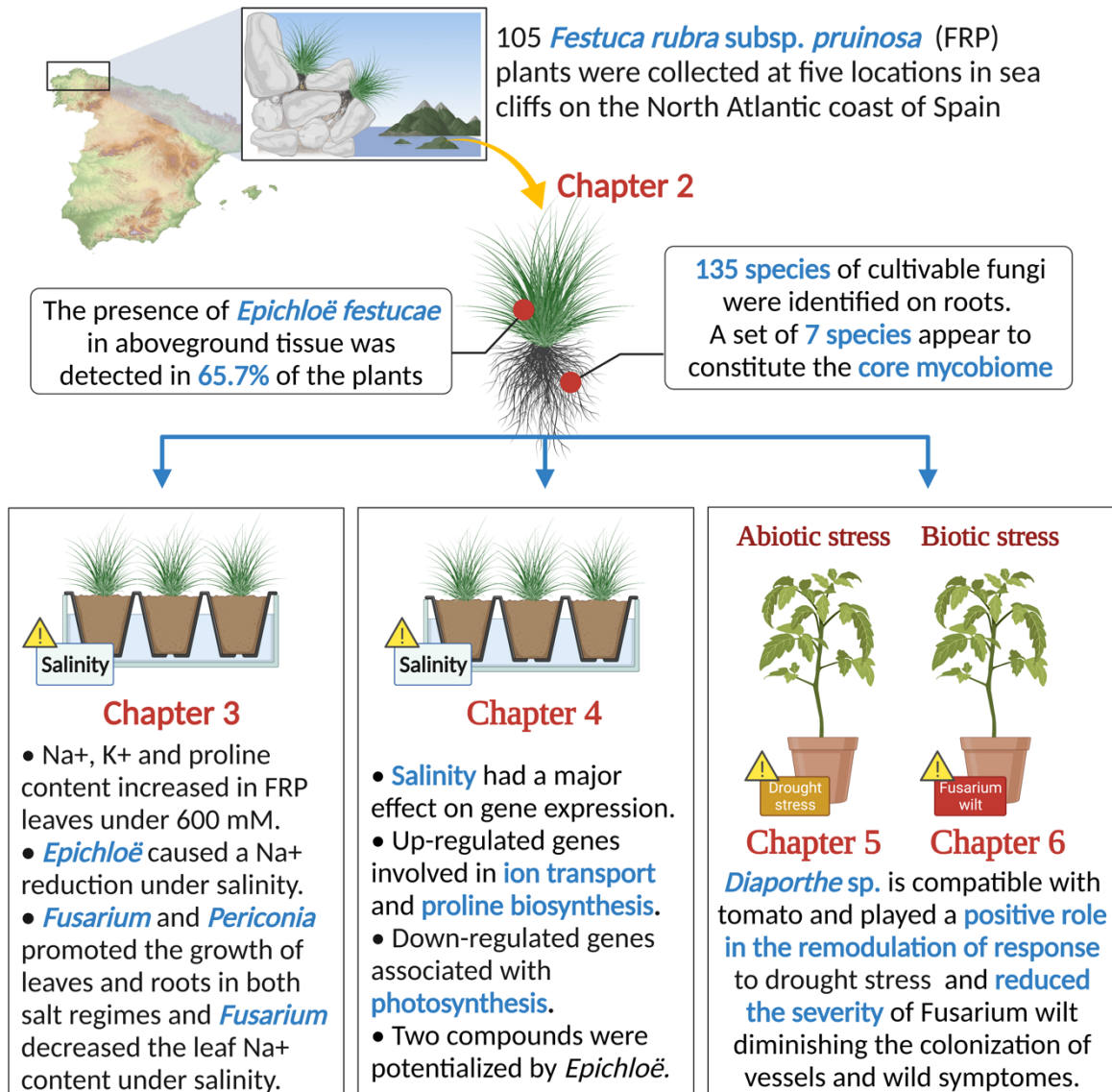

Figure 7.1 – General conclusions of the thesis

Publications

A Survey of Culturable Fungal Endophytes From *Festuca rubra* subsp. *pruinosa*, a Grass From Marine Cliffs, Reveals a Core Microbiome

Eric Pereira[1], Beatriz R. Vázquez de Aldana[1], Leticia San Emeterio[2] and Iñigo Zabalgogeazcoa[1]*

[1] *Institute of Natural Resources and Agrobiology of Salamanca, Consejo Superior de Investigaciones Científicas (IRNASA-CSIC), Salamanca, Spain,* [2] *Research Institute on Innovation & Sustainable Development in Food Chain (ISFood), Universidad Pública de Navarra, Pamplona, Spain*

Festuca rubra subsp. *pruinosa* is a perennial grass that inhabits sea cliffs of the Atlantic coasts of Europe. In this unhospitable environment plants grow in rock crevices and are exposed to abiotic stress factors such as low nutrient availability, wind, and salinity. *Festuca rubra* subsp. *pruinosa* is a host of the fungal endophyte *Epichloë festucae*, which colonizes aerial organs, but its root mycobiota is unknown. The culturable endophytic mycobiota of FRP roots was surveyed in a set of 105 plants sampled at five populations in marine cliffs from the northern coast of Spain. In total, 135 different fungal taxa were identified, 17 of them occurred in more than 10% of plants and in two or more populations. Seven taxa belonging to *Fusarium*, *Diaporthe*, Helotiales, *Drechslera*, *Slopeiomyces,* and *Penicillium* appeared to be constituents of the core microbiome of *Festuca rubra* subsp. *pruinosa* roots because they occurred in more than 20% of the plants analyzed, and at three or more populations. Most fungal strains analyzed (71.8%) were halotolerant. The presence of *Epichloë festucae* in aboveground tissue was detected in 65.7% of the plants, but its presence did not seem to significantly affect the structure of the core or other root microbiota, when compared to that of plants free of this endophyte. When plants of the grass *Lolium perenne* were inoculated with fungal strains obtained from *Festuca rubra* subsp. *pruinosa* roots, a *Diaporthe* strain significantly promoted leaf biomass production under normal and saline (200 mM NaCl) watering regimes. These results suggest that the core mycobiome of *Festuca rubra* subsp. *pruinosa* could have a role in host plant adaptation, and might be useful for the improvement of agricultural grasses.

Keywords: mycobiome, *Diaporthe*, *Fusarium oxysporum*, *Epichloë*, salinity, halophyte, grass

INTRODUCTION

The vegetation that inhabits coastal marine cliffs is adapted to environmental conditions that are far from optimal for plant growth and survival. The rock substrate and vertical cliffs makes soil scarce or non-existent. Sea water spray adds salinity to the scenario, and exposure to sea winds favor plant dehydration. Those conditions of low nutrient availability, salinity, and wind exposure

can be persistent in sea cliffs, and as a result, sea cliff vegetation is often endemic, reflecting habitat specialization in order to survive under these unhospitable conditions (Doody, 2001; López-Bedoya and Pérez-Alberti, 2009).

Festuca rubra subsp. *pruinosa* (FRP) is a plant species common in cliffs of the Atlantic coasts of Europe (Markgraf-Dannenberg, 1980; López-Bedoya and Pérez-Alberti, 2009). This perennial grass grows as a chasmophyte in rock fissures, or in very shallow soils formed on cliff cavities and slopes. In nature this species rarely occurs away from sea cliffs, where other vegetation predominates, and its salt tolerance is greater than that of other *F. rubra* subspecies adapted to inland habitats (Humpreys, 1982). Some anatomical characteristics might contribute to the adaptation to cliffs of this plant, for instance, the epithet *pruinosa* refers to the apparent epicuticular wax coat that covers its leaves, possibly having a role in preventing water loss (Ortuñez and de la Fuente, 2010; Martínez Sagarra et al., 2017).

In addition to traits inherent to the plant genome, the plant microbiome can also contribute to adaptation. Studies of some plants adapted to high stress habitats revealed that fungal endophytes confer habitat-specific stress tolerance to their hosts, and without these fungal endophytes plant adaptation is reduced in their native habitats (Rodriguez and Redman, 2008). Examples include improved tolerance to biotic and abiotic stress factors such as disease, herbivory, heat, or salinity mediated by endophytic fungi (Clay and Schardl, 2002; Waller et al., 2005; Rodriguez et al., 2008). Some of the endophytes reported in these studies conferred improved stress tolerance to new host species, highlighting the importance that endophytic fungi could have for the improvement of agricultural crops.

Like other subspecies of *Festuca rubra*, FRP plants maintain associations with the fungal endophyte *Epichloë festucae*. This fungus systemically colonizes the stems and leaves of host plants, but not the roots, and it is transmitted vertically to seeds (Leuchtmann et al., 1994; Zabalgogeazcoa et al., 2006). Endophytic *Epichloë* species can have a mutualistic relationship with their hosts, and increased tolerance of symbiotic plants to biotic and abiotic stress factors have been reported to occur in some situations. For example, *Epichloë festucae* can produce several types of alkaloids that might protect host plants against herbivores (Clay and Schardl, 2002).

In marine cliffs the roots of FRP plants grow in rock fissures or minimal soil, forming a compact fibrous system which holds the plant and captures nutrients. The root mycobiota of FRP is unknown, and some of its components could be useful for the improvement of other plant species of agronomic interest, as it has been demonstrated in other plant-endophyte associations (Rodriguez et al., 2008). Thus, the objectives of this work were: (1) to identify the culturable endophytic mycobiota of FRP roots, (2) to determine if the presence of *Epichloë* affects the structure of the root mycobiota, and (3) to test if some FRP root endophytes affect the performance of another grass, *Lolium perenne*, when exposed to salinity.

MATERIALS AND METHODS

Study Sites and Plant Sampling

Plants of *Festuca rubra* subsp. *pruinosa* (FRP) were collected at five locations in sea cliffs in the North Atlantic coast of Spain. Three locations were in Galicia: Torre de Hércules (TDH), 43°23′09″N 8°24′23″W, Cedeira (CED), 43°40′46″N 8°01′15″W, and Estaca de Bares (EDB), 43°47′25″N 7°41′16″W, and two in Asturias: San Pedro de la Rivera (SPR), 43°34′43″N 6°13′17″W, and Cabo de Peñas (CDP), 43°39′02″N 5°51′00″W. The shortest distance in straight line among these locations is 30 km. The predominant flora in the walls of these sea cliffs mainly consisted of *Festuca rubra* subsp. *pruinosa*, *Armeria* spp. and *Crithmum maritimum*. The climate in the coast of Galicia and Asturias is mild with oceanic influence and abundant rainfall spread over the year; during the 1981–2010 period the mean annual precipitation was 1106 and 1062 mm, and the average annual temperature 13.5 and 13.8°C in Galicia and Asturias, respectively (AEMET, 2012). In the spring of 2016, a total of 105 FRP plants, about 20 plants per location, were collected. Most plants grew in fissures in the rock, where soil was very scarce or absent. The plants were transported in a refrigerated cooler to the laboratory in Salamanca, and processed for the isolation of fungi from roots the day after they were sampled. Afterward the plants were transplanted to pots with a 1:1 (v:v) mixture of peat and perlite and maintained in a wirehouse outdoors.

Isolation of Fungi

To isolate fungi from roots, a sample of about 20 root fragments of 4–5 cm was collected from each plant. Each root sample was surface-disinfected with a solution of 20% commercial bleach (1% active chlorine) containing 0.02% Tween 80 (v:v) for 6 min, followed by treatment with an aqueous solution of 70% ethanol for 30 s. Finally, the roots were rinsed with sterile water and cut into pieces about 5 mm long. Thirty root pieces of each sample were plated in two Petri plates (15 pieces/plate) with potato dextrose agar (PDA) containing 200 mg/L of chloramphenicol. This antibiotic was used to exclude the isolation of endophytic bacteria. A root sample of each of the 105 plants was prepared as outlined above, and kept in the dark at room temperature. As mycelium emerged from a root fragment into the agar, a small piece of the mycelium from the leading edge of the colony was transferred to a new PDA plate and maintained at room temperature. The root fragment and remaining mycelium were taken out of the original plate to avoid overgrowth. The plates with root samples were checked daily for the presence of fungi for about 4 weeks.

The presence of *Epichloë festucae* on each plant was diagnosed by isolation. Several leaf sheaths were collected from each plant, cut into fragments about 5 mm long, and surface disinfected by immersion in a solution of 20% commercial bleach for 10 min. The fragments were then rinsed with sterile water, and about 15 fragments from each plant were placed in a PDA plate containing 200 mg/L of chloramphenicol. The plates were kept at room temperature, and fungi emerging from leaf fragments during the first 2–5 days were discarded together with its leaf sheath

fragment. White *Epichloë* mycelium emerging from the extremes of the leaf fragments about 1 week after plating was transferred to new PDA plates for further identification.

Identification of Fungi

The fungal isolates obtained from roots were first grouped into different morphotypes according to morphological characteristics such as colony color, exudate production, mycelium appearance, and growth rate. One or a few isolates of each morphotype were used for further classification based on rDNA nucleotide sequences. Fungal DNA was extracted from a small amount of mycelium scraped from a PDA culture using the Phire Plant Direct PCR Kit (Thermo Fisher Scientific). A ribosomal DNA region including the internal transcribed spacer 1 (ITS1), 5.8S rDNA, and ITS2 was amplified by PCR using primers ITS1 and ITS4 (White et al., 1990). Amplification conditions were: 98°C for 5 min, followed by 35 cycles of 98°C for 5 s, 54°C for 5 s, and 72°C for 20 s; after that the reaction was kept at 72°C for 1 min. PCR amplicons were cleaned (MSB Spin PCRapace, Stratec biomedical, Germany) and sequenced at the DNA sequencing service of the University of Salamanca (Spain).

All the sequences obtained were grouped into operational taxonomic units (OTU), considering that groups of sequences with a similarity greater than 97% belonged to the same OTU. This clustering operation was done using BlastClust software (NCBI, 2004). Afterward, a sequence representative of each OTU was used to search for similar curated sequences at the UNITE fungal database. A taxonomic identity was assigned to each OTU considering that the species rank of a UNITE database match was accepted when the identity between the OTU and database sequences was greater than 97%, and most UNITE matches corresponded to the same taxon. When the similarity was 97% – 95%, or UNITE matches corresponded to several species of the same genus, only the genus rank was accepted. In other cases the sequences were assigned to orders or families whenever it was reasonable.

Analysis of Root Fungal Diversity

For each location (referred to as population from here on), species accumulation curves showing the relationship between the number of plants sampled and the number of fungal species obtained, were estimated using the 'specaccum' function and the exact method with the Vegan Package in R (Oksanen et al., 2017). Estimations of the maximum number of fungal species at each population were obtained with the Bootstrap and Chao indexes using EstimateS 9.0 software (Colwell, 2005). Shannon's index of diversity (H') was estimated from the relative abundance of each taxon identified. The distribution of the relative abundance of the fungal species was observed with a rank-abundance curve. The similarity of fungal communities between each pair of populations was estimated using Jaccard's index of similarity (J). It is calculated from the equation $J = c/(a + b + c)$, where 'c' is the number of fungal taxa shared between two populations, 'a' the number of fungal taxa unique to the first population and 'b' the number of fungal taxa unique to the second population (Jaccard, 1912).

Effect of *Epichloë* on Root Mycobiota

Species richness (number of different root endophyte species per plant) was analyzed with a two-way ANOVA with *Epichloë* presence (E+) or absence (E−) and plant population (CED, CDP, EDB, SPR, and TDH) as factors. A type III sum of squares was used because the number of E+ and E− plants was unbalanced.

Species accumulation curves and beta diversity index estimations, plus a Canonical Correspondence Analysis (CCA) were made using the Vegan Package in R (Oksanen et al., 2017). Species accumulation curves for E+ and E− plants were estimated using the 'specaccum' function and the exact method. Beta-diversity indexes were estimated using the 'betadiver' function and the z index based on the Arrhenius species-area model (Koleff et al., 2003). Differences in beta diversity among groups were determined by Tukey multiple comparisons. A CCA was made because the gradient length of the detrended correspondence analysis (DCA) was greater than four, which indicated an unimodal response (Lepš and Šmilauer, 2003). Taxa appearing in less than three plants were omitted for this analysis; as a result, 61 taxa remained. A forward selection procedure (ordistep function) was used to determine the subset of explanatory variables (*Epichloë* incidence, population, *Epichloë*: population) explaining most variation in root mycobiome. The statistical power of the analysis was assessed by Monte Carlo permutation tests ($n = 999$).

Salt Tolerance of Fungal Isolates

A set of 46 fungal strains belonging to 20 of the most abundant genera isolated from FRP roots plus nine *Epichloë festucae* strains were analyzed to determine their salt tolerance *in vitro*. For each fungal strain a 6 mm diameter mycelial disk was placed in the center of 9 cm Petri plates with PDA containing three different concentrations of sodium chloride: 600 mM (equivalent to sea water concentration), 300 mM, and a control without NaCl. For each fungal strain and salt treatment three replicate plates were prepared. All plates were incubated at room temperature in the dark. The colony diameter was measured at two perpendicular axes when colonies in the fastest growing medium reached a diameter of 4–6 cm. The effect of salinity treatments on the radial growth of fungal colonies was assessed by means of a one-way ANOVA, and statistical significance of differences among means using Tukey's test ($p < 0.05$).

Extracellular Enzyme Activity

In vitro cellulase and amylase activity was analyzed for 43 strains belonging to some of the most abundant taxa. The production of cellulase was assayed using the method described by Sunitha et al. (2013) adapted to PDA plates. For each fungal strain a 6 mm diameter mycelial disk was placed in the center of a 9 cm. Petri plate and incubated for 5 days at 25°± 1°C in the dark. After incubation the plates were flooded with 0.2% (w/v) aqueous Congo Red, and distained with 1 M NaCl for 15 min. The presence of a clear zone surrounding the colony

indicated cellulase activity. Amylase activity was assessed on PDA containing 2% (w/v) soluble starch. After incubation the plates were flooded for 15 min with a solution of 1% (w/v) iodine in 2% (w/v) potassium iodide. A clear zone surrounding the colony indicated amylase activity (Hankin and Anagnostakis, 1975).

Inoculation of *Lolium perenne* Plants With Root Endophytes From FRP

To test whether FRP endophytes affect the growth of the grass *Lolium perenne* under salinity, plants were inoculated with three fungal strains belonging to some of the core taxa from FRP roots. A greenhouse experiment was conducted with a completely randomized design with 14 plant replicates for each fungal strain (*Periconia* S6, *Penicillium* E7, and *Diaporthe* S69) and salinity treatment (0 and 200 mM NaCl). Seeds of *Lolium perenne* cv. Tivoli (DLF, Denmark) were sown in 200 mL plastic pots filled with a substrate composed of seven parts of peat and perlite (1:1) previously sterilized at 80°C for 24 h, mixed with one part (v:v) of fungal inoculum. The fungal inoculum was a 4 week old culture of each fungus grown in autoclaved sugar beet pulp. Several seeds were sown in each pot, and thinned to four seedlings after emergence. Three weeks after germination, plants were watered with 0 or 200 mM NaCl during 3 weeks. Plants subject to the salinity treatment were watered with 50 and 100 mM NaCl on the first and third day respectively to avoid salt shock, and the 200 mM concentration was applied from day 5 onward. After 3 weeks of salt treatment the plants were harvested.

Five replicates of each treatment (salt and fungal strain) were analyzed for K and Na concentration by inductively coupled plasma atomic emission spectroscopy (ICP-OES, Varian 720-ES). Previously, dried plant samples were calcined at 450°C for 8 h, and ashes dissolved in $HCl:HNO_3:H_2O$ (1:1:8).

A two-way ANOVA was made to determine the effects of salt treatment and fungal strain on shoot biomass, K and Na concentrations, and differences between means were assessed using Tukey's test ($p < 0.05$). The success of the inoculation was determined after harvest by the reisolation of the inoculated fungi from surface disinfected roots, using the method above explained.

RESULTS

Endophyte Isolation

After plating 3150 root fragments on culture media, a total of 2324 fungal isolates were obtained, ranging from 355 to 578 among populations (**Table 1**). Most isolates emerged in the first 5 days after the placement of the roots on plates. Isolates were obtained from 73.8% of the root fragments plated. All sampled plants harbored fungi in their roots, and on average, 21 isolates were obtained from the roots of each plant.

Epichloë festucae was isolated from leaves of 65.7% of the plants. Its incidence among populations ranged from 20.0 to 100.0% (**Table 1**).

Identification of Fungal Isolates and Taxonomic Structure

When the isolates of each population were grouped according to morphotypes, the TDH isolates were classified into 177 morphotypes, CED in 142, EDB in 125, SPR in 137, and those from CDP in 107.

Nucleotide sequences were obtained from one or more isolates of each morphotype. As a result, 502 ITS1-5.8S-ITS2 nucleotide sequences were obtained, and those differing in homology by less than 3% were considered to belong to the same taxon. After this clustering process, 138 different sequences remained. These sequences were used to interrogate the UNITE sequence database, and as a result 135 fungal taxa were identified (**Supplementary Table S1**). Twenty-three taxa were identified to a species rank, 69 to genus rank and the remaining 43 were assigned to an order, class, family or division (**Table 2**). All the taxa could be assigned to 64 different fungal genera, 96% of them within the Ascomycota. Pleosporales, Hypocreales, and Eurotiales were the most representative orders, in terms of the number of taxa (23, 18, and 10%, respectively). The remaining orders were marginally represented (**Figure 1**). Among plant populations the number of fungal taxa ranged from 34 to 59 (**Table 1**).

The distribution of the taxa according to their incidence can be visualized in the rank-abundance curve shown in **Figure 2**. Seven species occurred in more than 20% of the plants at three

TABLE 1 | Incidence of *Epichloë* and fungal species richness in roots of *Festuca rubra* subsp. *pruinosa* at five populations from marine cliffs in Northern Spain.

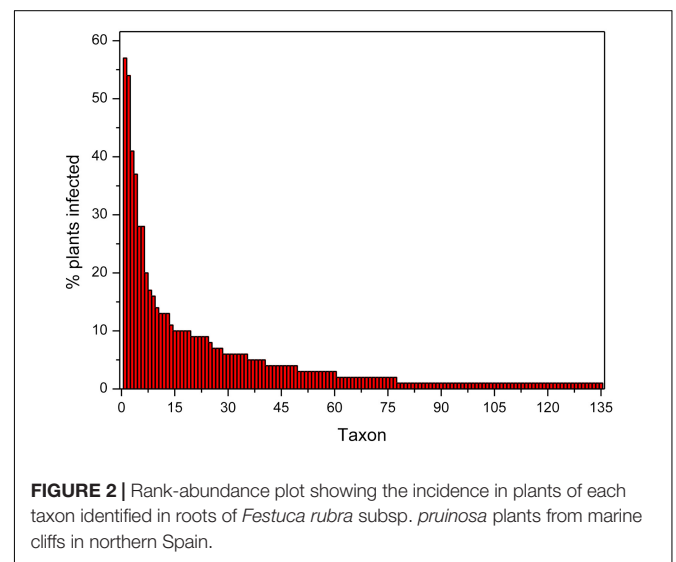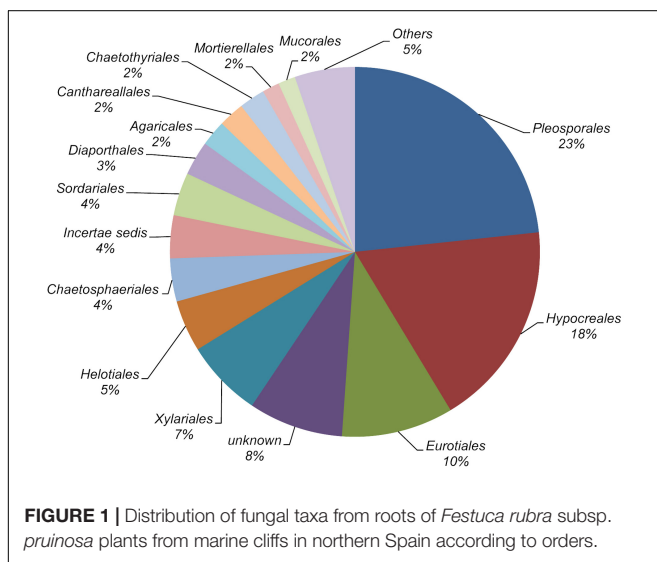| Population	Number of plants analyzed	Incidence of *Epichloë festucae* (%)	Root mycobiota			
			Number of isolates obtained	Colonization[1]	Number of fungal species	Fungal species per plant
TDH	21	57.1	471	74.8	34	1.62
CED	19	68.4	355	62.3	46	2.42
EDB	22	77.3	473	71.7	47	2.47
CDP	20	20.0	447	74.5	59	2.57
SPR	23	100.0	578	83.8	46	2.19
Total/mean	105	65.7	2324	73.8	135	1.29

[1]*Percentage of root pieces from which fungi emerged into growth medium.*

TABLE 2 | Core and abundant fungal species isolated from surface sterilized roots of *Festuca rubra* subsp. *pruinosa* at five populations from marine cliffs in northern Spain.

Strain	Taxon	Identity to closest match (%)	ITS sequence accession number	Order	Incidence in plants (%)	Number of populations
T150	*Fusarium oxysporum*	100	MH578626	Hypocreales	57.1	5
EB4	*Diaporthe* sp. A	100	MH578627	Diaporthales	54.3	5
C29	*Fusarium* sp. A	100	MH626490	Helotiales	41.0	4
S75	Helotiales sp. A	100	MH626491	Helotiales	37.1	5
T105	*Drechslera* sp.	100	MH626492	Pleosporales	27.6	4
S132	*Slopeiomyces cylindrosporus*	100	MH626493	Magnaporthales	27.6	3
T120	*Penicillium* sp. F	100	MH626494	Eurotiales	20.0	5
S7	*Darksidea* sp.	99	MH628220	Pleosporales	17.1	3
T131	*Periconia macrospinosa*	100	MH628221	Pleosporales	16.2	3
T122	*Penicillium* sp. A	100	MH628222	Eurotiales	14.3	4
T16	*Alternaria* sp. A	99	MH628223	Pleosporales	13.3	3
S38	*Fusarium* sp. B	99	MH628224	Hypocreales	13.3	4
C2	*Dactylonectria alcacerensis*	100	MH628225	Hypocreales	13.3	4
E79	Helotiales sp. B	100	MH628226	Helotiales	11.4	3
T140	*Alternaria* sp. B	100	MH628227	Pleosporales	10.5	4
E74	*Lachnum* sp. A	99	MH628228	Helotiales	10.5	3
CP17	*Trichoderma* sp. B	100	MH628229	Hypocreales	10.5	2

Only the taxa with an incidence in plants greater than 20% are listed. **Supplementary Table S1** *contains the complete list of 135 taxa identified.*

FIGURE 1 | Distribution of fungal taxa from roots of *Festuca rubra* subsp. *pruinosa* plants from marine cliffs in northern Spain according to orders.

FIGURE 2 | Rank-abundance plot showing the incidence in plants of each taxon identified in roots of *Festuca rubra* subsp. *pruinosa* plants from marine cliffs in northern Spain.

or more populations: *Fusarium oxysporum* (57.1%), *Diaporthe* sp. A (54.3%), *Fusarium* sp. A (40.9%), Helotiales sp. A (37.1%), *Slopeiomyces cylindrosporus* (27.6%), *Drechslera* sp. (27.6%), and *Penicillium* sp. F (20.0%) (**Table 2**). The identification of several *F. oxysporum* strains was confirmed by Martijn Rep and Maria Constantin (University of Amsterdam) by means of an analysis of their EF1α gene sequence. Because of their relatively high incidence within and among populations, these taxa could be considered as part of the core microbiome of FRP.

A second set of relatively abundant taxa were isolated from 10 to 20% of the plants, and at two or more populations (**Table 2**), these were *Darksidea* sp., *Periconia macrospinosa*, *Penicillium* sp.

A, *Alternaria* sp. A, *Fusarium sp. B*, *Dactylonectria alcacerensis*, Helotiales sp. B, *Alternaria* sp. B, *Lachnum* sp. A and *Trichoderma* sp. B. The remaining 118 taxa were found in less than 10% of the plants and 58 of them were singletons, occurring in a single plant.

Some of most abundant taxa, like *Darksidea* sp*., Periconia macrospinosa, Slopeiomyces cylindrosporus* and *Drechslera* sp., belong to the group of fungi known as dark septate endophytes (DSE). Fungi from the DSE group present some particular morphological characteristics, such as septated and melanized hyphae. These characteristics were observed in hyphae from two strains of Helotiales sp. A under the light microscope. Therefore, Helotiales sp. A also seems to belong to the DSE.

All populations produced non-asymptotic species accumulation curves, suggesting that increased sampling effort would reveal new fungal species (**Figure 3**). The Chao and Bootstrap estimators of the maximum number of species did not approach an horizontal asymptote, what made them unreliable estimators for this particular case.

Effect of *Epichloë festucae* on Root Endophytic Fungal Communities

In the set of 105 plants analyzed, 69 were infected by *Epichloë festucae* (E+) and 36 were not (E−). Out of the 135 fungal species identified in all plants, 52 were exclusive of E+ plants, 29 of E− plants, and 54 occurred in both.
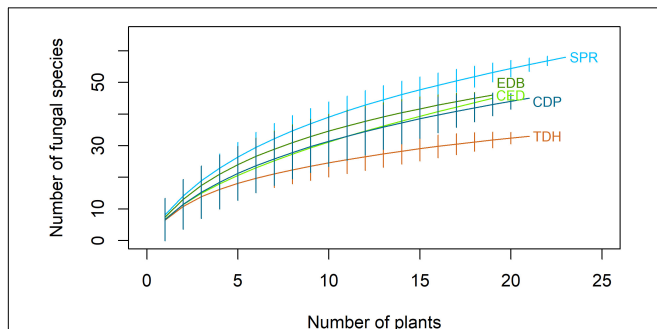
The ANOVA showed that neither the presence of *Epichloë* nor population had a significant effect on species richness ($F = 1.999$; $p = 0.276$ and $F = 1.626$; $p = 0.174$ respectively). The beta diversity index showed a similar trend, no significant differences were found between E+ and E− plants ($p = 0.989$) or among populations ($p = 0.377$ for all pairwise comparisons). The values of the Shannon diversity index (H') were relatively high, but similar for E+ and E− plants (**Table 3**).

Both E+ and E− plants displayed similar species accumulation curves when the data from all five populations were pooled (**Figure 4A**). The species richness accumulated at 36 plants was 80.93 ± 5.24 for E+ plants and 72.03 ± 1.04 for E− plants. Within each population, we found small differences (both positive and negative) between E+ and E− plants (**Figures 4B–F**).

The first two axes of the CCA were statistically significant ($p = 0.001$) and explained 35.18 and 29.36% of the variance. After the forward selection, only the variable population was finally included in the CCA and explained the 5.29% of the variation. The CCA biplot showed no clear separation between E+ and E− plants (**Figure 5**). However, there was a segregation among plant populations: the first axis clustered populations according to regions and separated the Asturian populations (CDP and SPR) from the Galician ones (TDH, CED and EDB); and the second axis segregated both Asturian populations, suggesting that the structure of the root mycobiota of these two populations differ between them and with respect to the Galician populations (**Figure 5**). All the core and the abundant taxa were present in both E+ and E− plants, although some species were more abundant in E+ (*Slopeiomyces cylindrosporus*) or in E− plants (*Drechslera* sp.) (**Figure 6**).

In terms of similarity of the fungal assemblages between pairs of populations, *J* values were higher between populations from the same region, 0.238 to 0.362 among Galician populations and 0.238 between Asturian populations, than between Galician and Asturian populations, which ranged from 0.095 to 0.193 (**Table 4**).
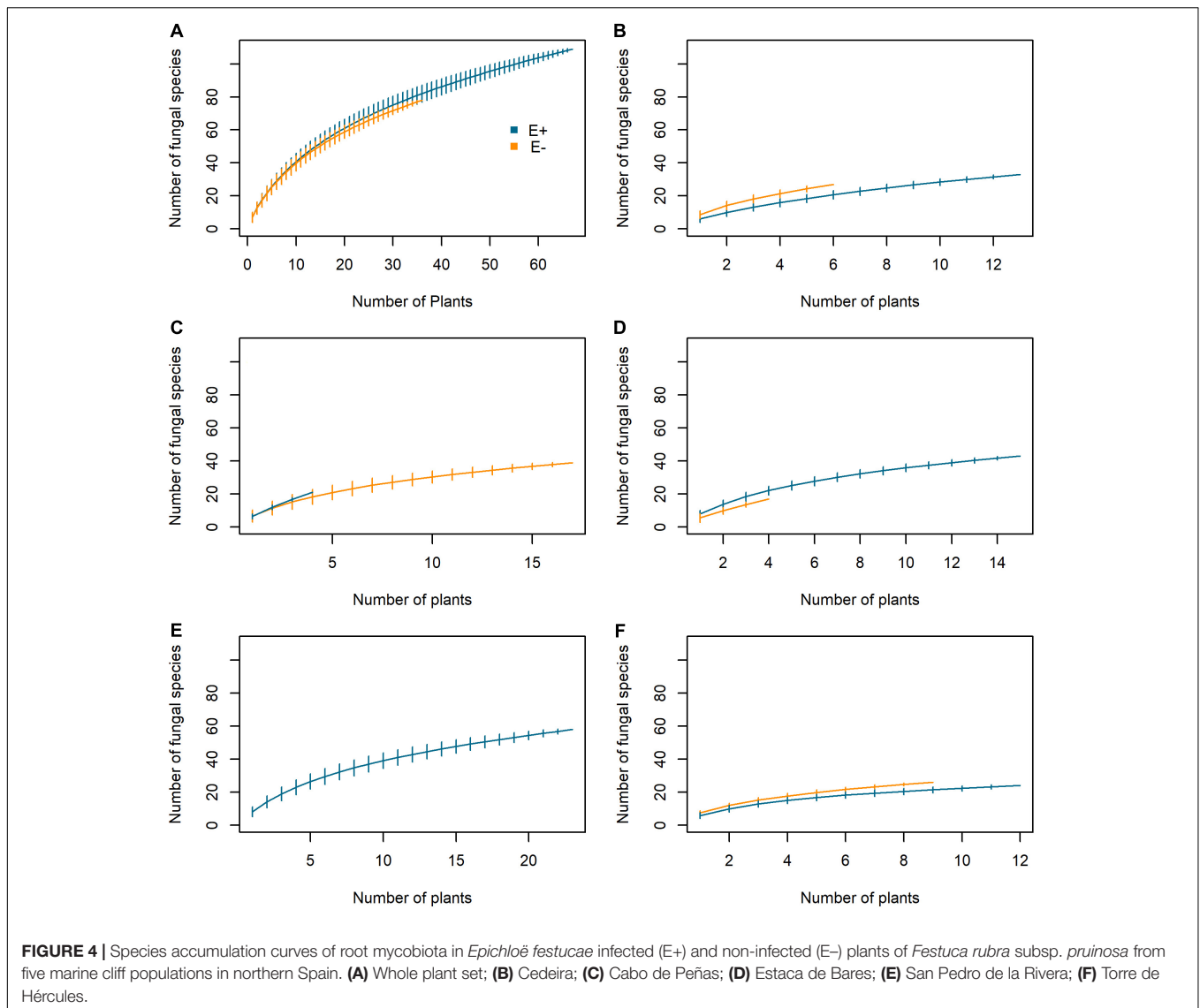
Salt Tolerance and Enzymatic Activity of Endophytic Fungi

The salt tolerance assay showed that fungal strains had three different types of response in terms of their radial growth. Most strains analyzed (71.8%) were halophilic, showing a statistically significant increase in radial growth in PDA plates containing NaCl respect to the control (**Supplementary Table S2**). The radial growth of 51.5% of these halophilic strains increased at both NaCl concentrations; that of 21.2% increased only in 600 mM NaCl, and that of 27.3% increased only in 300 mM NaCl. All nine *Fusarium* strains and four of the five *Diaporthe* sp. A strains tested were halophylic.

Some strains (6.5%) were halotolerant, not showing a significant difference in radial growth in 300 mM and 600 mM NaCl with respect to the control. Finally, 21.7% of the strains showed a radial growth decrease in culture media containing NaCl and were classified as halosensitive, 80.0% of these strains decreased only in 600mM NaCl, and the remaining 20.0% did it at both salt concentrations. Within taxa like *Diaporthe* sp. A, *Periconia macrospinosa* or *Penicillium* sp. F, some strains had different responses, i.e., *Diaporthe* strain S129 was halophilic and strain S69 halosensitive (**Supplementary Table S2**).

The nine *E. festucae* strains tested were halosensitive, all decreased in radial growth in the 600 mM medium (**Table 5**). Seven of them did not show a significant difference in radial growth with respect to the control at 300 mM NaCl.

Cellulase and amylase activities were assayed for 43 fungal strains (**Table 6**). Twenty three of these strains, including all tested strains of *Fusarium oxysporum*, *Penicillium* and Helotiales sp. A, showed cellulase activity *in vitro*. In contrast, none of the
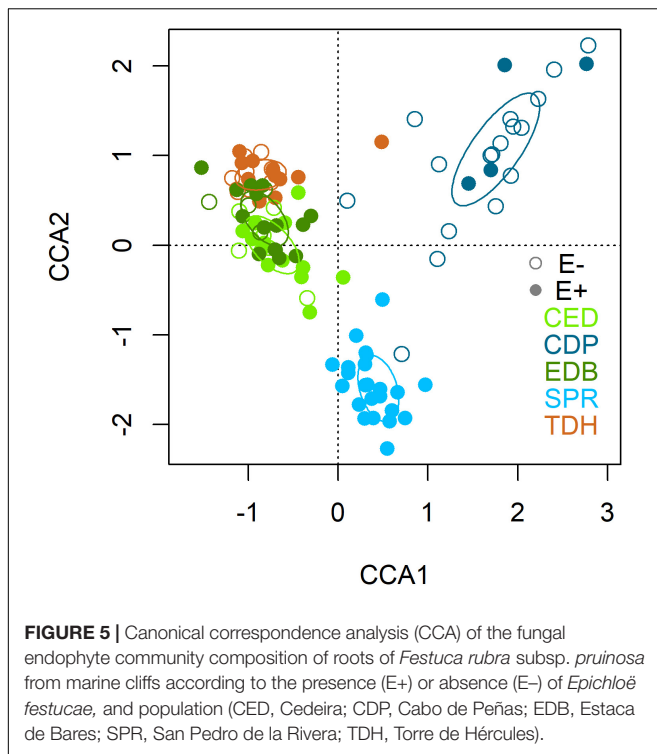

FIGURE 3 | Species accumulation curves for fungal species isolated from roots of *Festuca rubra* subsp. *pruinosa* at five populations from marine cliffs in northern Spain. TDH, Torre de Hércules; CED, Cedeira; EDB, Estaca de Bares; SPR, San Pedro de la Rivera; CDP, Cabo de Peñas.

TABLE 3 | Fungal species richness and diversity in roots of *Festuca rubra* subsp. *pruinosa* plants infected (E+) or not infected (E−) by *Epichloë festucae* at five populations in marine cliffs.

Factor		Number of plants analyzed	Species per plant	ß diversity (Kolleff)	H' Shannon
Epichloë	E+	69	7.19 ± 2.63	0.59 ± 0.07	4.04
	E−	36	7.08 ± 3.11	0.59 ± 0.08	3.90
Population	TDH	21	6.52 ± 1.91	0.51 ± 0.10	3.13
	CED	19	6.84 ± 2.59	0.50 ± 0.10	3.32
	EDB	22	7.47 ± 2.55	0.56 ± 0.10	3.48
	CDP	20	6.67 ± 3.45	0.55 ± 0.10	3.59
	SPR	23	8.17 ± 3.04	0.55 ± 0.05	3.43

FIGURE 4 | Species accumulation curves of root mycobiota in *Epichloë festucae* infected (E+) and non-infected (E−) plants of *Festuca rubra* subsp. *pruinosa* from five marine cliff populations in northern Spain. **(A)** Whole plant set; **(B)** Cedeira; **(C)** Cabo de Peñas; **(D)** Estaca de Bares; **(E)** San Pedro de la Rivera; **(F)** Torre de Hércules.

six *Diaporthe* sp. A strains tested was positive. Amylase activity was detected in only nine strains, including all four *Penicillium* strains tested.

Effect of FRP Endophytes on Growth of *Lolium perenne*

A two-way ANOVA showed a significant effect of salinity (p = 0.004; $\overline{X}_{control}$ = 0.236 g, $\overline{X}_{NaCl}$ = 0.192 g), endophyte inoculated (p < 0.001; $\overline{X}_{control}$ = 0.194 g, $\overline{X}_{Periconia}$ = 0.231 g, $\overline{X}_{Penicillium}$ = 0.109, $\overline{X}_{Diaporthe}$ = 0.321 g), and their interaction (p = 0.034; **Figure 7**) on dry matter production of *L. perenne*. Plants inoculated with *Diaporthe* S69, a *Diaporthe* sp. A strain, showed a significant increase in biomass production with respect to the uninoculated control plants in both watering treatments: 31.3% in tap water and 48.9% under saline irrigation (**Figure 7**). The plants inoculated with *Periconia* S6 had greater biomass

in both watering treatments, but the difference respect to the controls was not significant. In contrast, plants inoculated with *Penicillium* E7 did not show visual symptoms of stress such as dry leaves, but showed a significant decrease in biomass production under the tap water treatment; in the salinity treatment the difference in biomass was not significant with respect to uninoculated plants. In addition, the biomass of plants inoculated with *Penicillium* E7 did not differ between tap water and salinity treatments.

Sodium was significantly affected by salt (p < 0.001), endophyte inoculated (p < 0.001) and their interaction (p = 0.002). Inoculated plants with *Periconia* S6 and *Diaporthe* S69 strains had greater Na than controls under tap water treatment (**Figure 7**). When plants were salt irrigated, the increase in Na was greater in plants inoculated with E7, S6 or S69 strains than in control plants. Potassium content was significantly affected by salt (p = 0.038), endophyte inoculated (p < 0.001) and their interaction (p = 0.003). Inoculated plants with E7, S6 or S69

FIGURE 5 | Canonical correspondence analysis (CCA) of the fungal endophyte community composition of roots of *Festuca rubra* subsp. *pruinosa* from marine cliffs according to the presence (E+) or absence (E−) of *Epichloë festucae,* and population (CED, Cedeira; CDP, Cabo de Peñas; EDB, Estaca de Bares; SPR, San Pedro de la Rivera; TDH, Torre de Hércules).

strains had significantly greater K concentration than controls at water treatment (**Figure 7**). At salt treatment, plants inoculated with *Penicillium* E7 had the greatest K content.

After the harvest, root fragments of *Lolium perenne* were plated on culture media and the fungal isolates obtained were identified through morphological characteristics as the endophytes inoculated into the plants. The reisolation of these fungi indicated their compatibility with *L. perenne* and the success of plant inoculation.

DISCUSSION

The Core Microbiome of *Festuca rubra* subsp. *pruinosa*

The roots of *Festuca rubra* subsp. *pruinosa* were found to be a niche containing numerous fungal species, an assemblage of 135 culturable species was identified. This magnitude is not unusual in surveys of the mycobiota of grasses (Sánchez Márquez et al., 2012), but the high incidence of seven species that were present in more than 20% of the plants, and in several populations is remarkable. These species were *Fusarium oxysporum*, *Diaporthe* sp. A, *Fusarium* sp. A, Helotiales sp. A, *Drechslera* sp., *Slopeiomyces cylindrosporus,* and *Penicillium* sp. F. In particular, *Fusarium oxysporum* and *Diaporthe* sp. A occurred in more than 50% of the plants, and at all five populations examined. Those seven fungal species seem to be components of the core microbiome of FRP, because they are shared by a significant number of plants, and occur at different populations (Shade and Handelsman, 2012). It is not common to find a
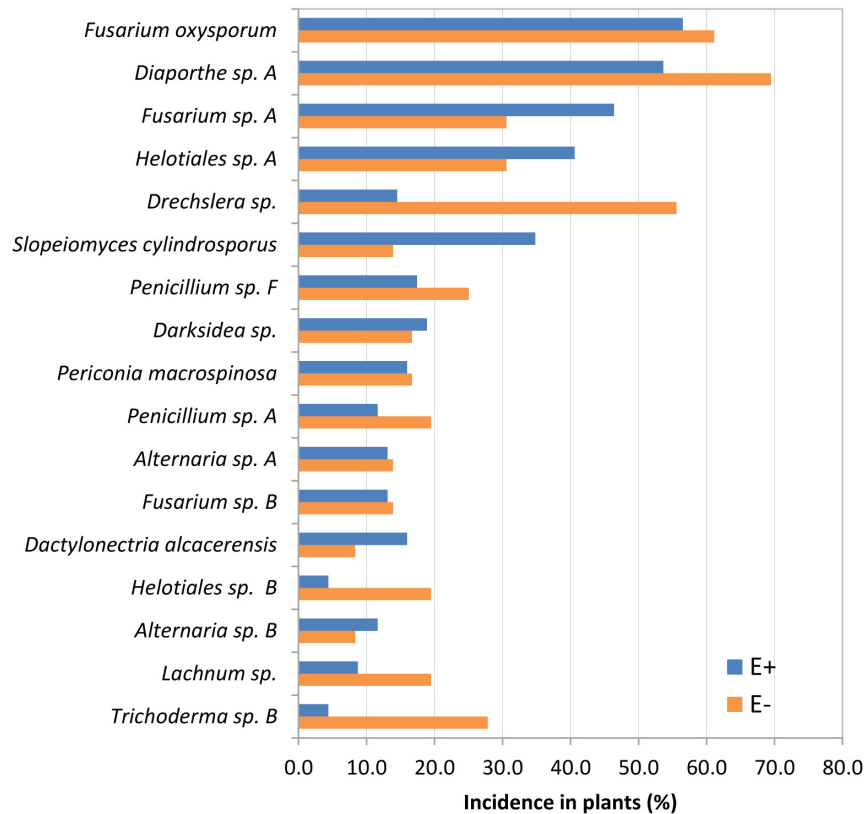
group of fungal species with such high incidence within and among plant populations. Using similar methodology, as well as culture independent methods, no more than two or three species with an incidence greater than 20% were found in surveys of other grasses (Sánchez Márquez et al., 2008, 2010; Ofek-Lalzar et al., 2016; Zhong et al., 2018). In addition, dominant species reported in several taxa of inland grasses, such as *Cladosporium* or *Epicoccum*, were absent from FRP plants (Peláez et al., 1998; Sánchez Márquez et al., 2012; Ofek-Lalzar et al., 2016).

Two of the core taxa of FRP belonged to the genus *Fusarium*. Although this genus is best known due to important pathogens of numerous agricultural species, it is also one of the most commonly isolated genera of endophytes from grasses and other plants (Vázquez de Aldana et al., 2013; Martins et al., 2016; Lofgren et al., 2018). Research on endophytic *Fusarium* has shown that some strains can improve the salinity tolerance of their host plants (Rodriguez and Redman, 2008; Redman et al., 2011). Furthermore, *F. oxysporum* strains obtained from FRP plants in this study were found to protect tomato plants against a pathogenic strain of *F. oxysporum* f.sp. *lycopersici* (Constantin et al., 2017).

The genus *Diaporthe* contains numerous species that behave as endophytes or pathogens, and in some cases as both, depending on the host plant species (Gomes et al., 2013). *Diaporthe* sp. A is a main component of the core microbiome of FRP, and species of this genus have also been reported as dominant components of the microbiome of olive and other plants (Martins et al., 2016; Noriler et al., 2018). Regarding mutualism, *Diaporthe* strains originally isolated from wild plant species promoted the growth of rice and tritordeum (Yang et al., 2015; Zabalgogeazcoa et al., 2018).

Our work revealed that associations between DSE and FRP roots are common in sea cliffs. Some of the core and most abundant taxa, such as *Darksidea* sp., *Periconia macrospinosa*, *Slopeiomyces cylindrosporus* and *Drechslera* sp., were previously reported as DSE in other grasses (Hornby et al., 1977; Knapp et al., 2012, 2015; Siless et al., 2018). In addition, Helotiales sp. A also seems to be a DSE because its hyphae had characteristics of this group, and other members of the Helotiales (i.e., *Phialocephala fortinii*) are recognized as DSE (Sieber and Grünig, 2013; Ridout et al., 2017). DSE colonize roots of plants communities in different habitats, and some authors hypothesized that these fungi can play an important role in plant adaptation to abiotic stress conditions, especially drought (Porras-Alfaro et al., 2008; Knapp et al., 2015). However, in spite of their abundance in nature, there is still uncertainty about the ecological significance of plant-DSE symbioses (Mandyam and Jumpponen, 2014).

Given the characteristics of the FRP habitat, strains from taxa belonging to the core microbiome of FRP are excellent candidates to test their possible role in host plant adaptation to salinity. Habitat-adapted symbiosis is a phenomenon which occurs when plants establish relationships with symbionts which enhance their adaptation to a particular stress factor present in their habitat (Rodriguez and Redman, 2008). Whether this occurs in the plant-endophyte systems here described would require inoculation of FRP seedlings and evaluation of plant performance

FIGURE 6 | Incidence in plants of *Festuca rubra* subsp. *pruinosa* from marine cliffs infected (E+) and not infected (E−) by *Epichloë festucae* of root species that constitute the core and abundant classes of the culturable mycobiome.

TABLE 4 | Jaccard index of similarity (bold) and number of fungal species identified in roots of each pair of populations (italic) of *Festuca rubra* subsp. *pruinosa* plants from marine cliffs.

Population	TDH	CED	EDB	SPR	CDP
TDH	**1.000**	0.238	0.362	0.095	0.147
CED	*63*	**1.000**	0.300	0.182	0.154
EDB	*58*	*70*	**1.000**	0.193	0.182
SPR	*84*	*88*	*88*	**1.000**	0.238
CDP	*68*	*78*	*77*	*84*	**1.000**

parameters under salinity stress. The search for endophytes from the core microbiome of wild plants adapted to unhospitable habitats has produced interesting solutions for the improvement of stress tolerance on agronomic crops (Redman et al., 2011; Ali et al., 2018).

Because of our research interest in culturable fungi, and the isolation methods used, components of the plant microbiome such as bacteria or non-culturable fungi were not identified in this survey. Members of these groups could have an important role in the adaptation of FRP plants to marine cliffs. For instance, symbioses with arbuscular mycorrizal fungi (AMF) can contribute to plant growth and protection under environmental stress (Lenoir et al., 2016). Symbiotic associations with AMF have been reported for some *Festuca* species (i.e.,

Dalpé and Aiken, 1998; Santos et al., 2006), but their presence and effects in FRP were not studied, and deserve attention.

In this work, about 72% of the fungal strains from FRP roots were classified as halophilic, their radial growth *in vitro* increased in the presence of NaCl. This category included some species of the core microbiome of FRP, like *Diaporthe* sp. A, *Fusarium oxysporum*, *Fusarium* sp. A, and Helotiales sp. A. In contrast*, E. festucae* showed a halosensitive response. The life cycle of this fungus which colonizes the intercellular space of aerial tissues and is seed transmitted, can be completely endophytic. Thus, host plants protect the fungus from the harmful saline environment. However, other fungal species which spend a part of their life cycle outside of their plant hosts might benefit from being halotolerant.
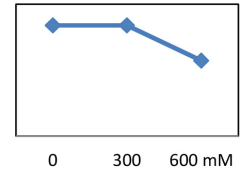
Cellulase or amylase enzymatic activity *in vitro* was detected in some of the core taxa, such as *Fusarium oxysporum,* Helotiales sp. A and *Penicillium* sp. F. These enzymes degrade cellulose and starch to soluble sugars such as glucose, cellobiose, and other oligomers which can be readily absorbed by plant roots (Carroll et al., 1983). Considering that FRP plants grow in rock fissures where soil and nutrients are very scarce, fungi with these enzymatic activities could have a role recycling nutrients from dead roots. However, these two enzymatic activities were not detected in cultures of *Slopeiomyces cylindrosporus*, a fungus with saprobic capability (Hornby et al., 1977), and cellulase activity

TABLE 5 | Radial growth of nine *Epichloë festucae* strains isolated from *Festuca rubra* subsp. *pruinosa* plants from marine cliffs in PDA plates with different NaCl concentrations.

Strain	Radial growth (cm)			Type of response	
	0 mM	300 mM	600 mM		
TDH1	2.12 ab	2.40 b	1.60 a	Halosensitive	
TDH11	1.97 ab	2.42 a	1.82 b	Halosensitive	
TDH3	2.67 a	2.60 a	1.62 b	Halosensitive	
CED6	2.67 a	2.17 ab	1.43 b	Halosensitive	
CED12	2.43 a	2.37 a	1.55 b	Halosensitive	
CED10	2.48 a	2.40 a	1.32 b	Halosensitive	
CED1	2.42 a	2.33 a	1.42 b	Halosensitive	
EDB9	2.37 a	1.25 b	0.67 c	Halosensitive	
EDB11	2.85 a	1.65 b	0.68 c	Halosensitive	

For each row different letters indicate significant differences at p < 0.05.

was absent form *Diaporthe* sp. A strains. This result could be due to non-induction of these enzymes in the culture medium used, because both fungal strains grew well as saprobes in a beet pulp medium, rich in carbohydrate and protein, which was used to prepare inoculum for plant inoculations.

Potential of FRP Endophytes for Plant Improvement

Knowledge about the role of endophytic fungi on plant adaptation to salinity stress is important because the world surface of saline soils is increasing, producing economic losses in crops (Munns and Gilliham, 2015). *Diaporthe* sp. A strain S69 improved the growth of plants of *Lolium perenne,* an important forage grass, in the presence and absence of salinity stress. On average, plants inoculated with *Diaporthe* S69 produced 31% more aerial biomass than the uninoculated controls under normal conditions, and 49% more under salinity stress. Similarly, fungal endophytes such as *Piriformospora indica*, *Fusarium culmorum*, or *Penicillium minioluteum* can alter physiological processes and improve tolerance to salt stress in agricultural crop species (Baltruschat et al., 2008; Khan et al., 2011; Redman et al., 2011).

One of the indirect consequences of salinity is an enrichment of Na and deficiency of K in plant cells, caused by the competition between Na and K, that have similar ionic radii and ion hydration energies (Munns and Tester, 2008). We found that *L. perenne* plants inoculated with *Periconia* S6, *Penicillium* E7 and *Diaporthe* S69 strains accumulated significantly more K in aboveground tissues under the tap water treatment than uninoculated plants; this suggests that an enrichment of Na due to salinity might have been prevented by the increased K content present before the stress. Similar results were observed in grasses inoculated with *Aspergillus aculeatus* (Xie et al., 2017) suggesting that the maintenance of a high level of K may contribute the alleviation of the negative effect of sodium. A beneficial effect of K accumulation in plants has also been reported for associations with arbuscular mycorrhiza (Langenfeld-Heyser et al., 2007) and *Epichloë* spp. (Chen et al., 2018). It is important to point out that the increase in biomass of *L. perenne* plants inoculated with

Diaporthe strain S69 occurred not only during salt treatment but also in the tap water treatment. This implies that the fungal effect improving plant growth was not a specific process induced by salinity. To study the effect of fungal strains on plant parameters which can be altered by endophytes to improve plant performance, such as phytohormones, photosynthetic capacity, nutrient absorption or antioxidant capability (Baltruschat et al., 2008; Redman et al., 2011; Leitão and Enguita, 2016) is a future objective of our research.

Effect of *Epichloë festucae*, an Aboveground Tissue Endophyte, on Root Mycobiota

The incidence of *Epichlöe festucae* in FRP populations was 65.7%, a value very similar to that of 69% observed in a previous survey that included the same populations from Galicia (Zabalgogeazcoa et al., 2006). The relatively high incidence of *E. festucae* suggests that in an unhospitable habitat like sea cliffs, the costs of harboring a systemic symbiont could be compensated by mutualism. However, endophyte incidences closer to 100% could be expected under such circumstances. Whether natural selection favoring E+ plants, the efficiency of seed transmission, or a combination of both processes are involved in the prevalence rates of *Epichloë* observed in FRP populations is unknown, and deserves further study. Imperfect seed transmission (<100%) has been reported in other grass – *Epichloë* systems (Gundel et al., 2009). High incidence of *Epichloë festucae* in *Festuca rubra* populations has been reported in semiarid grasslands (70%) (Zabalgogeazcoa et al., 1999), or in the Scottish islands of St. Kilda (80%) (Bazely et al., 1997). In contrast, in Finland only 9 of 49 infected *F. rubra* populations had frequencies greater than 50% (Wali et al., 2007), and no plants harboring *Epichloë* were found in populations from subarctic regions of Canada (Santangelo and Kotanen, 2016).
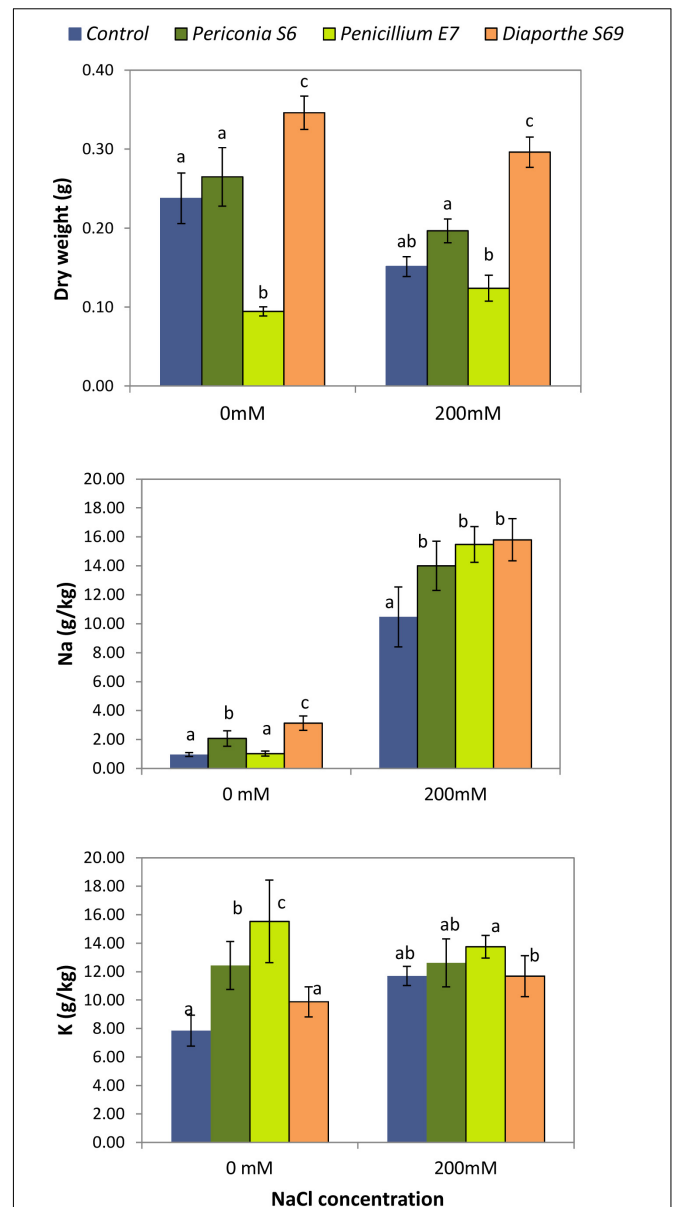
In some grass-endophyte associations *Epichloë* species could play a key role in salt tolerance. In pot experiments *Epichloë coenophiala* increased the root biomass of tall fescue (*Schedonorus arundinaceous*) (Sabzalian and Mirlohi, 2010), and another *Epichloë* species increased the shoot and root biomass

TABLE 6 | Cellulase and amylase activity in fungal strains isolated from roots *Festuca rubra* subsp. *pruinosa* plants from marine cliffs.

ID	Endophyte	Cellulase activity	Amylase activity
T16	*Alternaria* sp. *A*	−	++
C115	*Alternaria* sp. *B*	−	+
T90	*Codinaeopsis* sp.	++	−
C2	*Dactylonectria alcacerensis*	−	−
C1	*Darksidea* sp.	+	−
C7	*Darksidea* sp.	+	−
CP36	*Diaporthe* sp. A	−	−
EB4	*Diaporthe* sp. A	−	−
S129	*Diaporthe* sp. A	−	+
S32	*Diaporthe* sp. A	−	−
S69	*Diaporthe* sp. A	−	−
T18	*Diaporthe* sp. A	−	−
CP1	*Drechslera* sp.	−	−
E71	*Drechslera* sp.	−	−
T41	*Drechslera* sp.	−	−
T50	*Drechslera* sp.	−	−
CD8	*Epichloë festucae*	−	−
S13	*Fusarium* sp. A	+	−
T112	*Fusarium* sp. A	+	−
T6	*Fusarium* sp. A	+	−
C70	*Fusarium* sp. B	+	−
S38	*Fusarium* sp. B	+	+
CP3	*Fusarium oxysporum*	++	−
S10	*Fusarium oxysporum*	+	++
SP8	*Fusarium oxysporum*	+	−
T150	*Fusarium oxysporum*	+	−
E79	Helotiales sp. B	+++	−
S74	*Lachnum* sp.	−	−
C44	Helotiales sp. A	++	−
S75	Helotiales sp. A	++	−
T141	Helotiales sp. A	++	−
T29	Helotiales sp. A	+	−
T3	Helotiales sp. A	++	−
T114	*Penicillium* sp. F	++	+++
C13	*Penicillium* sp. A	+	+
E7	*Penicillium* sp. A	+++	+
T59	*Penicillium* sp. A	++	+
S6	*Periconia macrospinosa*	−	−
T131	*Periconia macrospinosa*	−	−
C43	*Slopeiomyces cylindrosporus*	−	−
S5	*Slopeiomyces cylindrosporus*	−	−
T70	*Slopeiomyces cylindrosporus*	−	−
CP17	*Trichoderma* sp. B	++	−

(−) No enzymatic activity; (+) Slight activity; halo < 3 mm. (++) Moderate activity; halo < 5 mm. (+++) High activity; halo > 5 mm.

FIGURE 7 | Effect of inoculation with strains *Periconia* S6, *Penicillium* E7 and *Diaporthe* S69, isolated from *Festuca rubra* subsp. *pruinosa*, on dry matter production, and Na and K content of *Lolium perenne* plants watered with 0 mM and 200 mM NaCl. For each NaCl concentration, different letters indicate significantly different means ($p < 0.05$).

of wild barley (*Hordeum brevisubulatum*) under salinity stress (Song et al., 2015; Chen et al., 2018). In contrast, in FRP plants no significant effect of *Epichloë* on shoot dry weight was detected under salt treatment, although root growth or other parameters that could be affected by the presence of *E. festucae* under salinity were not analyzed (Zabalgogeazcoa et al., 2006). Nevertheless,

in a stressful habitat like sea cliffs, environmental pressure on a holobiont might not necessarily affect an individual endophyte, but an assemblage where interactions among the plant host and the eukaryotic and prokaryotic microbiome components might be complex.

The presence of *Epichloë* in aboveground tissues of the host plant can affect underground processes by altering rhizospheric conditions that affect the density and activity of soil microorganisms (Omacini et al., 2012). This may result from endophyte effects on root exudates that can act as

chemical attractants or repellents in the rhizosphere (Malinowski et al., 1998). For instance, phenolic compounds are microbial inhibitors, and they increase in roots due to the presence of *Epichloë* (Ponce et al., 2009; Vázquez de Aldana et al., 2011). The effect of *Epichloë* on arbuscular mycorrhizal fungi has been extensively studied, and reduction, promotion, and null effects have been reported (Omacini et al., 2006; Novas et al., 2012; Rojas et al., 2016). Our results indicate that *E. festucae* did not have a clear and significant effect on the composition of the core microbiome or other mycobiota from FRP roots, although changes in the abundance of some species were found. These results are in agreement with other studies where the presence of *Epichloë* did not alter fungal colonization in roots (Vandegrift et al., 2015; Slaughter and McCulley, 2016) or shoots (Zabalgogeazcoa et al., 2013). Nevertheless, Zhong et al. (2018) reported that the presence of *Epichloë* decreased the diversity of root-associated fungi in *Achnatherum inebrians* and changed the community composition. However, such changes were in fungal orders with an abundance lower than 10%, where the number of isolates of these taxa can be low.

CONCLUSION

In conclusion, this study shows that numerous species of culturable fungi are associated to the roots of *Festuca rubra* subsp. *pruinosa* in its sea cliff habitat. Within this fungal assemblage of 135 species, a set of seven species occurred in a relatively high number of plants and locations, and those seem to be components of the core mycobiome of FRP: *Fusarium oxysporum*, *Diaporthe* sp. A, *Fusarium* sp. A, Helotiales sp. A, *Drechslera* sp., *Slopeiomyces cylindrosporus,* and *Penicillium* sp. F. Strains of these species are very promising candidates to study their role in the adaptation of FRP plants to salinity, a characteristic stress factor of their habitat. Furthermore, a *Diaporthe* strain belonging to the core taxa significantly improved the growth of *Lolium perenne* plants under normal and salinity stress conditions, showing the potential of the FRP core microbiome for the improvement of agricultural crops.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.03321/full#supplementary-material

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The Role of Fungal Microbiome Components on the Adaptation to Salinity of *Festuca rubra* subsp. *pruinosa*

Eric C. Pereira, Beatriz R. Vazquez de Aldana, Juan B. Arellano and
Iñigo Zabalgogeazcoa*

Plant-Microorganism Interaction Research Group, Institute of Natural Resources and Agrobiology of Salamanca, Consejo Superior de Investigaciones Científicas (IRNASA-CSIC), Salamanca, Spain

Festuca rubra subsp. *pruinosa* is a perennial grass that inhabits sea cliffs, a habitat where salinity and low nutrient availability occur. These plants have a rich fungal microbiome, and particularly common are their associations with *Epichloë festucae* in aboveground tissues and with *Fusarium oxysporum* and *Periconia macrospinosa* in roots. In this study, we hypothesized that these fungi could affect the performance of *F. rubra* plants under salinity, being important complements for plant habitat adaptation. Two lines of *F. rubra*, each one consisting of *Epichloë*-infected and *Epichloë*-free clones, were inoculated with the root endophytes (*F. oxysporum* and *P. macrospinosa*) and subjected to a salinity treatment. Under salinity, plants symbiotic with *Epichloë* had lower Na^+ content than non-symbiotic plants, but this effect was not translated into plant growth. *P. macrospinosa* promoted leaf and root growth in the presence and absence of salinity, and *F. oxysporum* promoted leaf and root growth in the presence and absence of salinity, plus a decrease in leaf Na^+ content under salinity. The growth responses could be due to functions related to improved nutrient acquisition, while the reduction of Na^+ content might be associated with salinity tolerance and plant survival in the long term. Each of these three components of the *F. rubra* core mycobiome contributed with different functions, which are beneficial and complementary for plant adaptation to its habitat in sea cliffs. Although our results do not support an obvious role of *Epichloë* itself in FRP salt tolerance, there is evidence that *Epichloë* can interact with root endophytes, affecting host plant performance.

Keywords: endophytes, *Epichloë*, fungi, *Fusarium oxysporum*, halophyte, *Periconia macrospinosa*, salinity, symbiosis

INTRODUCTION

Festuca rubra is a perennial grass that occurs in very diverse ecological niches (Markgraf-Dannenberg, 1980; Inda et al., 2008). In addition to its value as a forage, commercial cultivars of this species are used for ornamental and sports lawns (Braun et al., 2020). Within *F. rubra*, several subspecies have been defined and three of them, *litoralis*, *pruinosa*, and *arenaria*, occur in maritime habitats such as salt marshes, sea cliffs, or coastal sands

(Markgraf-Dannenberg, 1980). As a result of habitat adaptation, maritime subspecies are more tolerant to salinity than inland subspecies (Hannon and Bradshaw, 1968; Rozema et al., 1978). *Festuca rubra* subsp. *pruinosa* (FRP) inhabits sea cliffs of the Atlantic coasts of Europe (**Figure 1**; Markgraf-Dannenberg, 1980). This grass often grows as a chasmophyte in rock crevices with low nutrient availability and high exposure to seawater spray and desiccating winds. Some structural traits seemingly associated with salt tolerance in FRP are a dense layer of epicuticular wax which covers its leaves, stomata enclosed on the adaxial side of c-sectioned leaves, together with a thickened root endodermis in comparison with inland fescues (Baumeister and Merten, 1981; Ortuñez and de la Fuente, 2010; Martinez-Segarra et al., 2017).

In addition to the plant traits that may favor tolerance to salinity and water loss, or improve nutrient absorption in a suboptimal environment, the plant microbiome can also provide auxiliary functions that facilitate the habitat adaptation of holobionts (i.e., the host plant and its microbiota; Rodriguez et al., 2008; Vandenkoornhuyse et al., 2015; Trivedi et al., 2020). In a previous study, 135 different fungal taxa were identified as culturable components of the fungal mycobiome of FRP roots (Pereira et al., 2019). *Fusarium oxysporum* was the most abundant taxon, being found at all populations sampled and in 57% of the plants analyzed. *Periconia macrospinosa,* a dark septate endophyte (DSE), was also an abundant component of the root microbiome, and it was found in 16% of the plants. In addition, aerial tissues of 66% of the FRP plants were colonized by the fungal endophyte *Epichloë festucae*. As possible components of the core microbiome of FRP, we here hypothesize that these fungi (*F. oxysporum, P. macrospinosa,* and *E. festucae*) could provide functions related to plant adaptation to the sea cliff environment.

Epichloë festucae asymptomatically colonizes the intercellular space of stem and leaf tissues of *F. rubra* and other grasses, and is vertically transmitted to seeds (Clay and Schardl, 2002; Leuchtmann et al., 2014). This fungal endophyte might produce antiherbivore secondary metabolites, such as the bioactive alkaloid ergovaline in symbiotic FRP plants (Vázquez de Aldana et al., 2007). Although *Epichloë* endophytes are not present in plant roots, their presence in aboveground plant tissues can affect several belowground processes (Omacini et al., 2012; Rojas et al., 2016). *Epichloë* is one of the best known taxa of endophytic fungi in aboveground tissues, but for most components of root mycobiomes, their functions as plant symbionts, as well as their life cycles, are unknown (Pozo et al., 2021).

Soil salinity inhibits plant growth and development by reducing water uptake, and also induces cytotoxicity due to excess of Na^+ ions, oxidative stress due to the generation of reactive oxygen species, and nutritional imbalance (Munns and Tester, 2008; Zhao et al., 2020). Plants, particularly halophytes, have mechanisms involved in adaptation to salinity, like Na^+ exclusion, Na^+ intracellular accumulation and osmoregulation, or alteration of the level of secondary metabolites, including phenolic compounds and their antioxidant capacity (Munns and Tester, 2008; Waśkiewicz et al., 2013;

van Zelm et al., 2020; Zhao et al., 2020). In addition, there is compelling evidence of fungal microbiome components contributing to plant adaptation to salinity. For example, some strains of *Epichloë*, *Diaporthe*, *Piriformospora*, *Penicillium*, *Fusarium*, and DSE have been reported to improve plant growth under salinity (Waller et al., 2005; Rodriguez et al., 2008; Molina-Montenegro et al., 2018; Pereira et al., 2019; Gonzalez Mateu et al., 2020; Wang et al., 2020).

The main purpose of this study was to explore the effect of the foliar endophyte *E. festucae* and the root endophytes *F. oxysporum* and *P. macrospinosa* on the performance of FRP plants subjected to salinity. We present data supporting that these microbiome components could be involved in the host plant adaptation to its maritime habitat.

MATERIALS AND METHODS

Plant and Fungal Material

Two near-isogenic lines of FRP (TH12 and EB15) were used to test the effects of *E. festucae* and two root endophytes on plant performance. Each line consisted of a unique plant genotype infected (E⁺) or not infected (E⁻) by a unique *E. festucae* genotype. Each line was generated from a single E⁺ FRP plant originally collected in the coast of Galicia (**Figure 1**; Pereira et al., 2019). This plant was split into several clones that were transplanted to 200 ml pots containing a 1:1 (v:v) mixture of peat and perlite. To obtain E⁻ plants, one half of the E⁺ clones were treated with the systemic fungicide propiconazole to eliminate the fungus (Zabalgogeazcoa et al., 2006a). Six doses of 400 μg of propiconazole were applied to each plant: The first, fourth, fifth, and sixth doses were applied to the soil, and the second and third were foliar applications. Fungicide treatments were spaced by 5 days for the first three treatments and by 10 days for the last three soil applications. Four weeks after the last dose, newly formed ramets from each clone were obtained, and their E⁺ or E⁻ status was verified by direct isolation of *E. festucae* from surface-disinfected leaf sheaths (Florea et al., 2015). Two different near-isogenic lines of FRP were used because different *Epichloë*/grass genotypes might differ in terms of stress responses, nutrient accumulation, or alkaloid content (Cheplick et al., 2000; Zabalgogeazcoa et al., 2006a; Vázquez de Aldana et al., 2020b). Only one root endophyte was inoculated into each *Festuca* line because of limited plant clone availability.

The fungal strains, *F. oxysporum* T48 and *P. macrospinosa* T131, were originally isolated as endophytes from surface-disinfected roots of asymptomatic FRP plants collected in natural populations in the northern coast of Galicia, Spain (Pereira et al., 2019). These strains were selected because they belonged to two of the most abundant taxa from the culturable fungal microbiome of roots from apparently healthy FRP plants (Pereira et al., 2019). Thus, we assumed that these microbiome components were non-pathogenic and could have a role in holobiont adaptation. In addition, *F. oxysporum* strains from FRP are non-pathogenic on tomato plants, as shown by Constatin et al. (2020).

Effect of *Epichloë* and Root Endophytes on Plant Growth Under Salinity

To determine the effect of *F. oxysporum* T48 and *P. macrospinosa* T131 (onward *F. oxysporum* and *P. macrospinosa*) on the growth of E^+ and E^- FRP plants, a greenhouse experiment was designed. Plants of the line EB15 were inoculated with *F. oxysporum*, and those of the line TH12 with *P. macrospinosa*. Each clone to be inoculated with a root endophyte was transplanted to a 200 ml pot containing a substrate composed of seven parts (v:v) of a mixture of peat and perlite (1:1 v/v) previously treated at 80°C for 24 h, and one part of fungal inoculum prepared in a beet pulp medium for 4 weeks (Vázquez de Aldana et al., 2020a). Non-inoculated clones were transplanted to pots containing only the peat and perlite substrate. A three-factor experiment was carried out for each line, with *Epichloë* infection (E^+ or E^-), root endophyte inoculation with *F. oxysporum* or *P. macrospinosa* (uninoculated or inoculated), and salinity (watering with 0 or 600 mM NaCl), with five plant replicates per treatment. Plants subjected to the salinity treatment were watered with 200 mM NaCl on the first day to avoid salt shock and with 600 mM NaCl afterward for 5 weeks. After this time, the whole plants were harvested, roots were carefully washed with tap water, and some pieces from each plant were kept for observation by optical microscopy. The plants were lyophilized, and their dry biomass was recorded. The aboveground parts, which consisted of leaves and leaf sheaths, were ground and used for chemical analysis.

To check for the presence of *F. oxysporum* or *P. macrospinosa* in roots of all inoculated plants, fresh root fragments were cleared in 5% KOH at 90°C for 15 min, neutralized with approximately three volumes of 1% HCl at 20°C overnight, stained with trypan blue (Berthelot et al., 2016), and visualized by light microscopy.

Sodium, Potassium, and Proline Content

To estimate the concentration of mineral elements, leaf samples (five replicates of each treatment) were calcined at 450°C for 8 h and ashes were dissolved in $HCl:HNO_3:H_2O$ (1:1:8). Na and K contents were analyzed by inductively coupled plasma atomic emission spectroscopy (ICP-OES) in a Varian 720-ES (Agilent, United States) spectrometer.

Leaf proline content was quantified in three plant replicates of each treatment using the spectrophotometric method described by Shabnam et al. (2016), adapted to 96-well plates in our laboratory. Approximately 15 mg of plant material was homogenized in 500 µl of 3% aqueous 5-sulfosalicylic acid and kept for 10 min in ice. The mixture was centrifuged at 10°C and 16,000 *g* for 10 min, and the supernatant was mixed with 250 µl of glacial acetic and 500 µl of ninhydrin reagent. Then, the mixture was heated at 99°C for 40 min and immediately cooled in ice. The mixture was centrifuged, and an aliquot of 200 µl transferred to a 96-well plate where the absorbance was measured at 513 nm in a FLUOstar Omega plate reader (BMG Labtech, Germany). L(−) proline (Acrós Organics) was used as standard for quantification.

Ferric Reducing Antioxidant Potential Assay

The total antioxidant capacity was determined in leaves of five replicates of each treatment using the ferric ion reducing antioxidant power (FRAP) method (Benzie and Strain, 1996). This method is based on the reduction of the colorless $[Fe(III)-,4,6-tri(2-pyridyl)-s-triazine)_2]^{3+}$ complex, abbreviated as Fe(III)-TPTZ, to the blue-colored Fe(II)-TPTZ complex, formed by the action of electron donating antioxidants at low pH. The FRAP reagent was prepared by mixing 300 mM acetate buffer pH = 3.6, a solution of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl, and 20.35 mM $FeCl_3$ at a volume ratio of 10:1:1. 5 mg of each plant sample was extracted in 700 µl of 50% aqueous acetone for 30 min in an ultrasound bath at 8°C. The mixture was centrifuged and transferred to a 96-well plate where 8 µl of sample, 8 µl of phosphate buffer saline (PBS), and 200 µl of FRAP reagent were added to each well. The absorbance was measured at 593 nm after 30 min in a FLUOstar Omega (BMG Labtech, Germany). A standard curve was prepared using different concentrations of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). The TPTZ solution was freshly prepared before use. The results were expressed as µmol Trolox equivalent per gram of dry weight.

Phenolic Compounds Content

The content of total phenolic compounds in leaf samples was determined spectrophotometrically according to the Folin-Ciocalteu method (Ainsworth and Gillespie, 2007). For the analyses, five replicates of each treatment were used. A 100 µl aliquot of acetone extract of each sample, prepared as previously described for the FRAP assay, was mixed with 500 µl of Folin-Ciocalteu reagent (Scharlab Chemie SA). After 5 min, a volume of 400 µl of a 700 mM Na_2CO_3 solution was added. The mixture was incubated for 60 min, and the absorbance at 765 nm was measured in a 96-well plate in a FLUOstar Omega (BMG Labtech, Germany). Gallic acid was used as a reference standard, and the results were expressed as µmol gallic acid equivalent per gram of dry weight.

Ergovaline Content

The *Epichloë* alkaloid ergovaline was analyzed in leaf samples of E^+ plants (three replicates of each treatment) by HPLC following a modification of the methods described by Hill et al. (1993) and Yue et al. (2000). Extraction was conducted in 0.5 g of plant material, adding 10 ml of chloroform, 0.5 ml of a methanolic solution 5 mM NaOH, and an internal standard of ergotamine (Sigma-Aldrich). The mixture was placed on an orbital shaker at 100 rpm for 120 min, paper-filtered (Filter Lab 1240), washed with 3.0 ml of chloroform, and then passed through an Ergosil – HL silica gel (500 mg, Analtech) column preconditioned with 5.0 ml of chloroform. To eliminate pigments, a solution of 5.0 ml chloroform:acetone (75:25 v/v) was passed through the column, and the sample was eluted with 3.0 ml of methanol and dried under a nitrogen stream. The residue was dissolved in 1.0 ml of methanol and filtered through a 0.45 µm nylon disk. Ergovaline quantification was

performed in a Waters 2695 HPLC system, with a C18 column (150 × 4.6 mm; 2.7 μm; Agilent Poroshell) and a fluorescence detector (Waters 2475). The excitation and emission wavelengths were 250 nm and 420 nm, respectively. The mobile phase was acetonitrile: 0.01 M ammonium acetate (3:7) with a gradient flow of 0.6 ml/min. The gradient was adjusted through time programming as follows: step 1, 30% acetonitrile and 70% ammonium acetate for 18 min; step 2, 45%:55% for 2 min; step 3, 85%:15% for 2 min; step 4, 100% acetonitrile for 5 min; step 5, 100% acetonitrile for 3 min; and step 6, 30%:70% for 2 min. The ergovaline standard was purchased from Forrest Smith (Auburn University, United States).

In vitro Dual-Culture Interaction

A dual-culture assay was made to analyze *in vitro* interactions between *E. festucae* and both root endophytes. The *E. festucae* strain from E⁺ plants of the line TH12 was co-cultured with *P. macrospinosa*, and the *E. festucae* strain from EB15 plants with *F. oxysporum*. Mycelial disks (6 mm diameter) from PDA cultures of each fungus were placed 4 cm apart on the surface of 9 cm Petri dishes containing PDA medium. Controls consisted of agar plates containing two disks of the same strain. Five replicates of each combination were incubated at room temperature in the dark. The evolution of the radii of the interacting strains was measured for several days. Interactions between strains were assessed based on the inhibition or non-inhibition of their mycelial growth.

Statistical Analyses

The effects of *E. festucae*, root endophyte inoculation, and salinity on biomass, Na⁺ and K⁺ content, proline, total phenolic compounds, and antioxidant activity were analyzed by means of a three-way ANOVA. For ergovaline concentration in E⁺ plants, the effects of root endophyte inoculation and salt treatment were analyzed with a two-way ANOVA. The data sets were evaluated for the statistical assumptions of the ANOVA with the Shapiro-Wilk normality test and Brown-Forsythe equal variance test. Differences between means of estimated effects of significant factors and their interaction were evaluated using Tukey's test ($p < 0.05$). All statistical analyses were performed with SigmaPlot v.14.

RESULTS

Biomass Production

The leaf biomass of the *Festuca* line EB15 was significantly affected by salinity, *Epichloë*, *Fusarium*, and the [*Epichloë* × *Fusarium*] interaction (**Table 1**). Leaf biomass decreased with salinity, E⁺ clones had greater biomass than E⁻ clones regardless of salinity, and inoculation with *Fusarium* increased the leaf biomass in all treatments, but this response was greater in E⁻ than in E⁺ plants (**Figure 2A**). In addition, root biomass was significantly greater in plants inoculated with *Fusarium* (0.818 ± 0.051 g vs. 0.647 ± 0.050 g) regardless of *Epichloë* infection or the salinity treatment (**Table 1**; **Figure 2B**).

In *Festuca* line TH12, only *Periconia* had a significant effect on leaf biomass (**Table 1**). Inoculated plants (0.536 ± 0.049 g) had greater leaf biomass than uninoculated (0.331 ± 0.028 g), regardless of *Epichloë* and salinity (**Figure 2C**). Root biomass was significantly affected by *Periconia,* [*Epichloë* × salt] and [*Epichloë* × *Periconia* × salt] interactions (**Table 1**). The triple interaction indicated that the positive effect of *Periconia* on root growth was more pronounced in E⁺ plants at 0 mM NaCl and in E⁻ plants at 600 mM NaCl (**Figure 2D**).

Sodium and Potassium Content

In both FRP lines, a significant effect of salinity, both root endophytes, and the interactions [*Epichloë* × root endophyte], [root endophyte × salinity], and [*Epichloë* × root endophyte × salinity] on Na⁺ content of leaves were detected (**Table 1**). In both plant lines, Na⁺ content increased in plants treated with 600 mM NaCl, and in uninoculated plants, it was lower in E⁺ than in E⁻ clones (**Figures 3A,D**). In *Festuca* line EB15, the Na⁺ content under salinity decreased significantly in plants inoculated with *Fusarium,* but it was affected by the *Epichloë* status. In line TH12, the opposite occurred, and at 600 mM NaCl, the Na⁺ content increased in E⁺ plants inoculated with *Periconia*; however, in E⁻ plants, differences between inoculation treatments were not significant (**Figure 3D**).

In both plant lines, the K⁺ content significantly increased at 600 mM NaCl, although this increase was less pronounced than the one observed for Na⁺ (**Figures 3B,E**). Neither the *Epichloë* status nor the root endophytes significantly affected this parameter (**Table 1**).

Mainly driven by the Na⁺ response, the Na⁺/K⁺ ratio significantly increased in leaves of all plants at 600 mM NaCl. In both lines, a significant effect of root endophyte inoculation [*Fusarium* and *Periconia*] and the interactions [*Epichloë* × root endophyte], [root endophyte × salinity], and [*Epichloë* × root endophyte × salinity] were detected (**Table 1**). In uninoculated plants of the line EB15 subject to salinity, this ratio was significantly lower in E⁺ than in E⁻ plants, and in plants inoculated with *Fusarium,* Na⁺/K⁺ further decreased in both E⁺ and E⁻ (**Figure 3C**). In line TH12, the Na⁺/K⁺ was also lower in uninoculated E⁺ plants at 600 mM NaCl, and the inoculation with *Periconia* did not reduce this ratio, as observed with *Fusarium* in the other line (**Figure 3F**).

Proline Content

Salinity had a significant effect on the leaf proline content in both FRP lines (**Table 1**; **Figure 4**). Neither *Epichloë* nor the root endophytes affected the content of this osmolyte. The proline content in leaves at 600 mM NaCl was very similar in both lines (EB15: 5.606 ± 0.440 g/kg; TH12: 5.664 ± 0.193 g/kg).

Antioxidant Capacity and Phenolic Compounds Content

Both salinity and *Fusarium* factors had a significant effect on the antioxidant capacity and content of total phenolic compounds (TPhC; **Table 1**; **Figure 5**). Plants inoculated with

TABLE 1 | ANOVA results showing the effect of *Epichloë* presence, inoculation, and salt treatment on different parameters of *F. rubra* subsp. *pruinosa* plants.

		Epichloë (E)	Root endophyte (R)	Salt	R × Salt	E × R	E × Salt	E × R × Salt
Shoot biomass								
Line EB15/*F. oxysporum* T48	$F_{7,39}$	6.08	29.5	4.64	1.65	8.06	0.632	0.061
	p	**0.019**	**<0.001**	**0.039**	0.208	**0.008**	0.432	0.808
Line TH12/*P. macrospinosa* T131	$F_{7,39}$	0.03	14.3	3.78	2.42	0.092	0.429	1.78
	p	0.862	**<0.001**	0.061	0.129	0.763	0.517	0.191
Root biomass								
Line EB15/*F. oxysporum* T48	$F_{7,39}$	1.65	5.51	0.004	0.464	0.079	2.89	0.005
	p	0.207	**0.025**	0.951	0.500	0.780	0.099	0.940
Line TH12/*P. macrospinosa* T131	$F_{7,39}$	0.362	20.3	1.67	0.514	0.001	7.44	5.67
	p	0.552	**<0.001**	0.205	0.478	0.980	**0.010**	**0.023**
Proline								
Line EB15/*F. oxysporum* T48	$F_{7,23}$	2.04	0.246	187	0.437	0.953	2.44	0.869
	p	0.175	0.628	**<0.001**	0.519	0.346	0.141	0.367
Line TH12/*P. macrospinosa* T131	$F_{7,23}$	0.418	1.21	694	0.101	0.705	0.262	0.783
	p	0.529	0.291	**<0.001**	0.756	0.416	0.617	0.392
Na								
Line EB15/*F. oxysporum* T48	$F_{7,39}$	1.69	40.1	193	41.1	10.6	2.01	9.16
	p	0.214	**<0.001**	**<0.001**	**<0.001**	**0.006**	0.178	**0.009**
Line TH12/*P. macrospinosa* T131	$F_{7,39}$	0.128	9.25	622	8.45	6.9	0.379	6.75
	p	0.726	**0.009**	**<0.001**	**0.012**	**0.021**	0.549	**0.022**
K								
Line EB15/*F. oxysporum* T48	$F_{7,39}$	0.429	0.005	25.5	0.096	0.005	0.663	0.663
	p	0.523	0.946	**<0.001**	0.761	0.944	0.429	0.429
Line TH12/*P. macrospinosa* T131	$F_{7,39}$	0.357	0.163	24.4	0.682	0	1.31	0.001
	p	0.561	0.693	**<0.001**	0.424	0.997	0.273	0.988
Na:K								
Line EB15/*F. oxysporum* T48	$F_{7,39}$	3.74	86.2	414	87.6	25.6	4.06	22.1
	p	0.074	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.064	**<0.001**
Line TH12/*P. macrospinosa* T131	$F_{7,39}$	0.355	8.12	319	7.44	4.97	0.146	4.87
	p	0.561	**0.014**	**<0.001**	**0.017**	**0.044**	0.709	**0.046**
Antioxidant capacity								
Line EB15/*F. oxysporum* T48	$F_{7,39}$	6.08	29.5	4.64	1.65	8.06	0.63	0.06
	p	**0.019**	**<0.001**	**0.039**	0.208	**0.008**	0.432	0.808
Line TH12/*P. macrospinosa* T131	$F_{7,39}$	0.03	14.3	3.78	2.42	0.092	0.429	1.78
	p	0.862	**<0.001**	0.061	0.129	0.763	0.517	0.191
Phenolic compounds								
Line EB15/*F. oxysporum* T48	$F_{7,39}$	1.65	5.51	0.004	0.464	0.079	2.89	0.005
	p	0.207	**0.025**	0.951	0.500	0.78	0.099	0.940
Line TH12/*P. macrospinosa* T131	$F_{7,39}$	0.362	20.3	1.67	0.514	0.001	7.44	5.67
	p	0.552	**<0.001**	0.205	0.478	0.98	**0.010**	**0.023**

Statistically significant values (p < 0.05) are shown in bold type.

Fusarium had lower antioxidant capacity and TPhC content than uninoculated plants, and both parameters decreased with salinity. *Fusarium* had a significant interaction with *Epichloë*, and in inoculated plants, the antioxidant capacity was lower in E⁺ than in E⁻ plants.

The antioxidant capacity of FRP line TH12 was significantly affected by salinity and the [*Epichloë* × salinity] interaction (**Table 1**; **Figure 5**), decreased with salinity in E⁻ plants, but in E⁺ plants, differences between salt treatments were not statistically significant. Inoculation with *Periconia* was the only factor affecting the TPhC content, which significantly decreased in plants infected with the root endophyte, regardless of the *Epichloë* infection and the salt treatment (**Table 1**; **Figure 5**).

Ergovaline Content

Epichloë festucae can produce ergovaline in symbiotic FRP plants. Therefore, this alkaloid was analyzed only in E⁺ plants of each line. Plants inoculated with *Periconia* or uninoculated had ergovaline contents below the limit of quantification (0.02 µg/g), suggesting that this FRP-*Epichloë* combination does not produce ergovaline. In plants of line EB15, there was a significant positive effect of salinity ($F = 6.02$, $p = 0.044$) on ergovaline content, but the effect of *Fusarium* was not statistically significant (**Figure 6**).

FIGURE 1 | *Festuca rubra* subsp. *pruinosa* (FRP) inhabits rocky sea cliffs of the Atlantic coasts of Europe (left). Plants often grow in rock fissures, where soil is absent, and are very exposed to wind and saline sea spray (right).
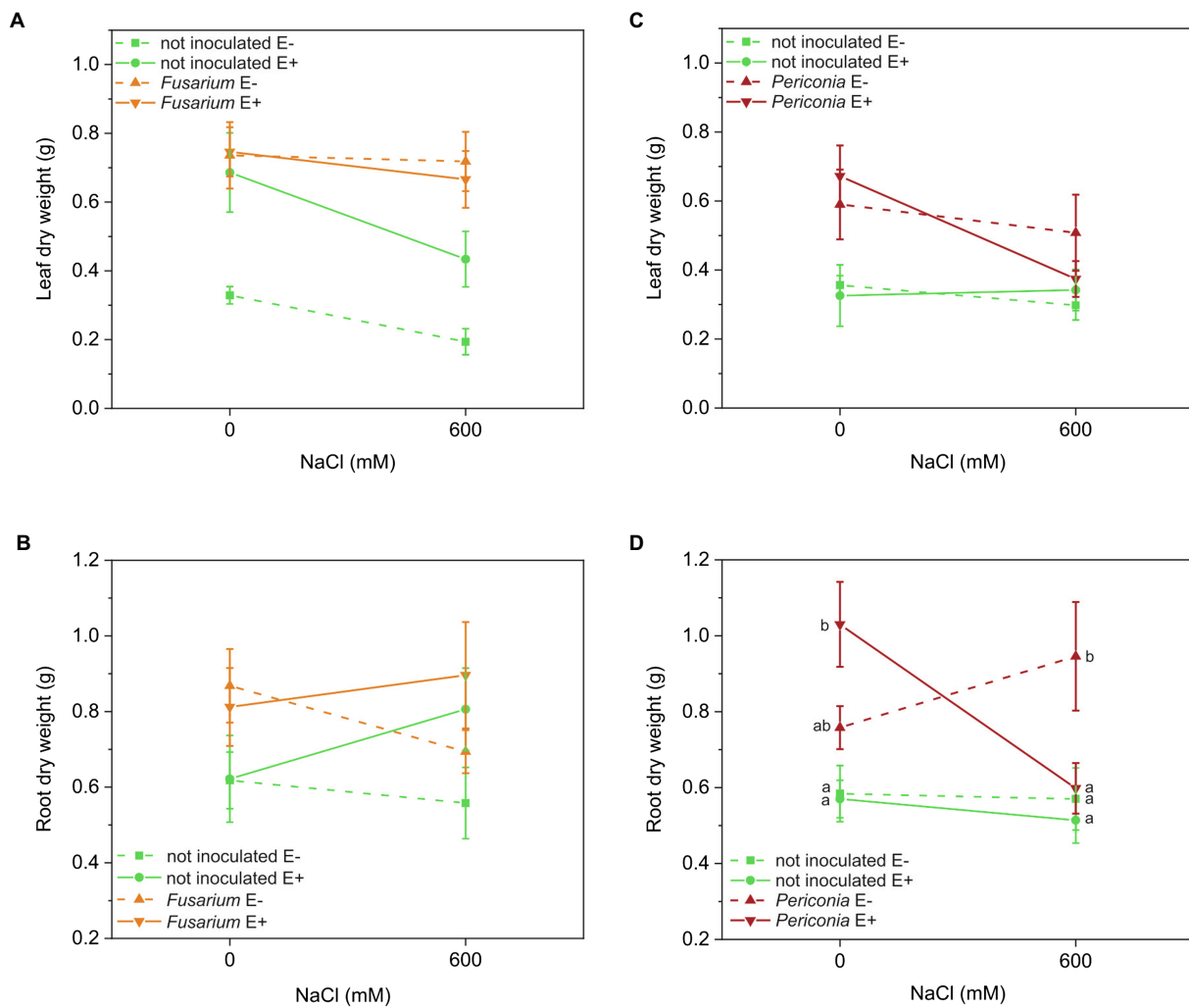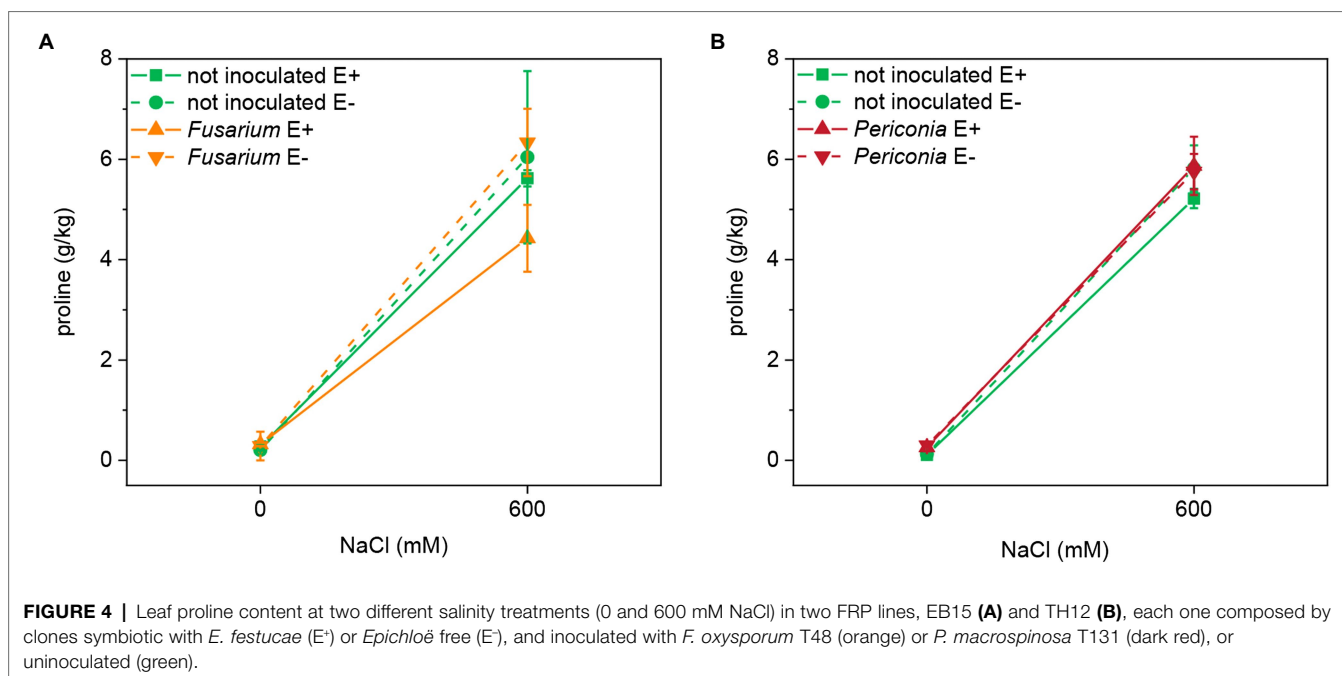

FIGURE 2 | Leaf and root biomass produced at two different salinity treatments (0 and 600 mM NaCl) in two FRP lines, EB15 **(A,B)** and TH12 **(C,D)**, each one composed by clones symbiotic with *Epichloë festucae* (E+) or *Epichloë* free (E−), and inoculated with *Fusarium oxysporum* T48 (orange), *Periconia macrospinosa* T131 (dark red), or uninoculated (green). Where a significant [salinity × *Epichloë* × root endophyte] interaction occurred, different means are indicated by different letters.
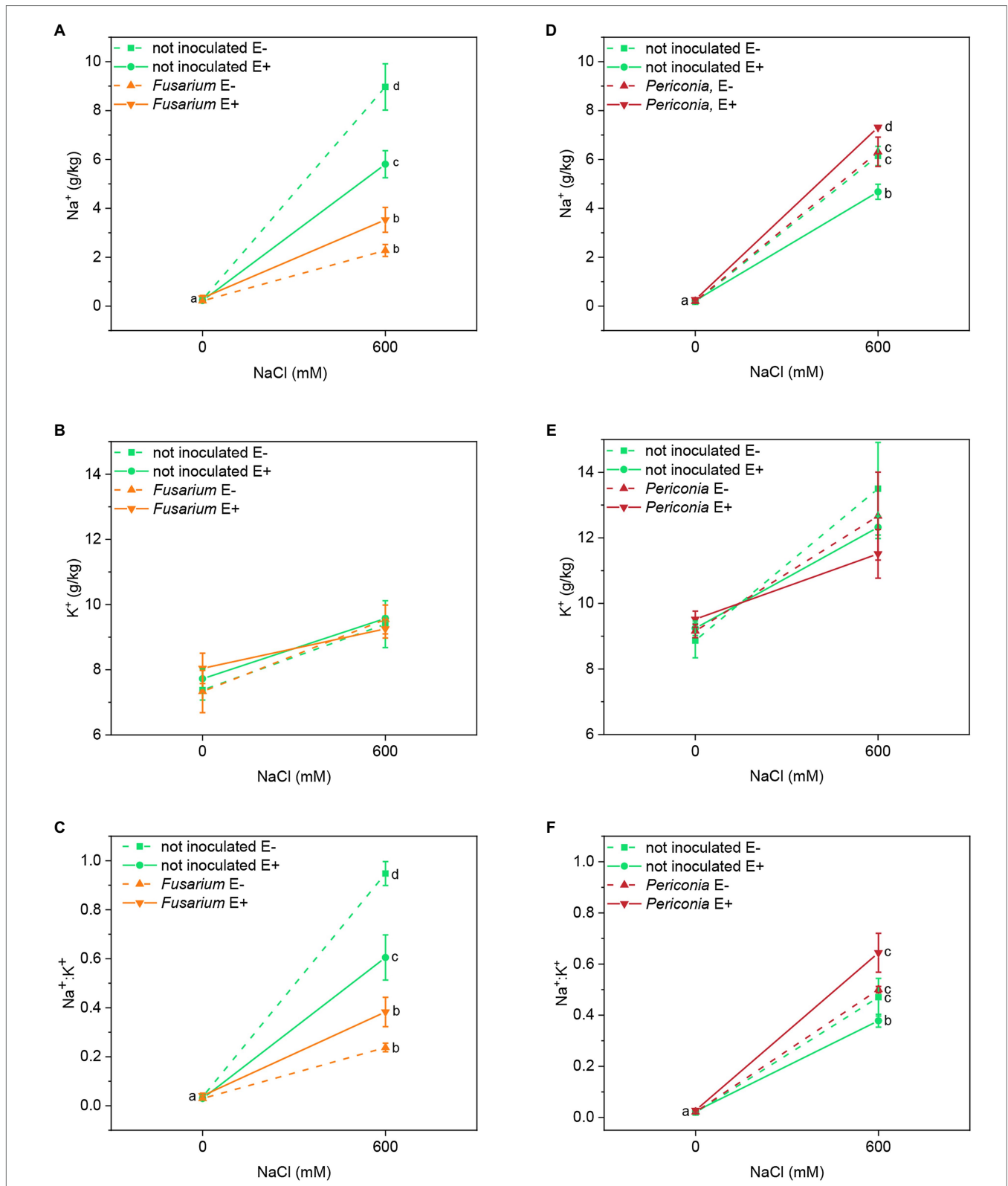
FIGURE 3 | Leaf sodium and potassium content and Na⁺:K⁺ ratio in two FRP lines, EB15 **(A–C)** and TH12 **(D–F)**. Each line was composed by clones symbiotic with *E. festucae* (E⁺) or *Epichloë* free (E⁻). Plants of each line were inoculated with *F. oxysporum* T48 (orange), *P. macrospinosa* T131 (dark red), or uninoculated (green), and subjected to two different salinity treatments (0 and 600 mM NaCl). Where a significant [salinity × *Epichloë* × root endophyte] interaction occurred, different means are indicated by different letters.

FIGURE 4 | Leaf proline content at two different salinity treatments (0 and 600 mM NaCl) in two FRP lines, EB15 **(A)** and TH12 **(B)**, each one composed by clones symbiotic with *E. festucae* (E⁺) or *Epichloë* free (E⁻), and inoculated with *F. oxysporum* T48 (orange) or *P. macrospinosa* T131 (dark red), or uninoculated (green).

Root Microscopy

The presence of fungal structures in inter and intracellular spaces indicated the presence of the root endophytes and successful plant inoculation (**Figure 7**). In roots of plants inoculated with *Periconia*, melanized septate hyphae and microsclerotia were observed (**Figures 7A,B**). These structures are typical of DSEs. In plants inoculated with *Fusarium* T48, hyphae were observed in the root cortex (**Figures 7C,D**).

In vitro Interaction Between *Epichloë festucae* and Root Endophytes

Dual cultures of *E. festucae* strains isolated from the FRP lines and the root endophytes *P. macrospinosa* and *F. oxysporum* were established. The results showed that *E. festucae* had an inhibitory effect on the mycelial growth of *P. macrospinosa* (**Figure 8A**). However, this pattern was not observed with *F. oxysporum*, whose mycelium grew over the *E. festucae* colony (**Figure 8B**).

In dual cultures with *Periconia* and *Fusarium*, the radial growth of *E. festucae* was stimulated, increasing by 42 and 48%, respectively, relative to the dual cultures of *E. festucae* alone (**Figures 8C,D**).
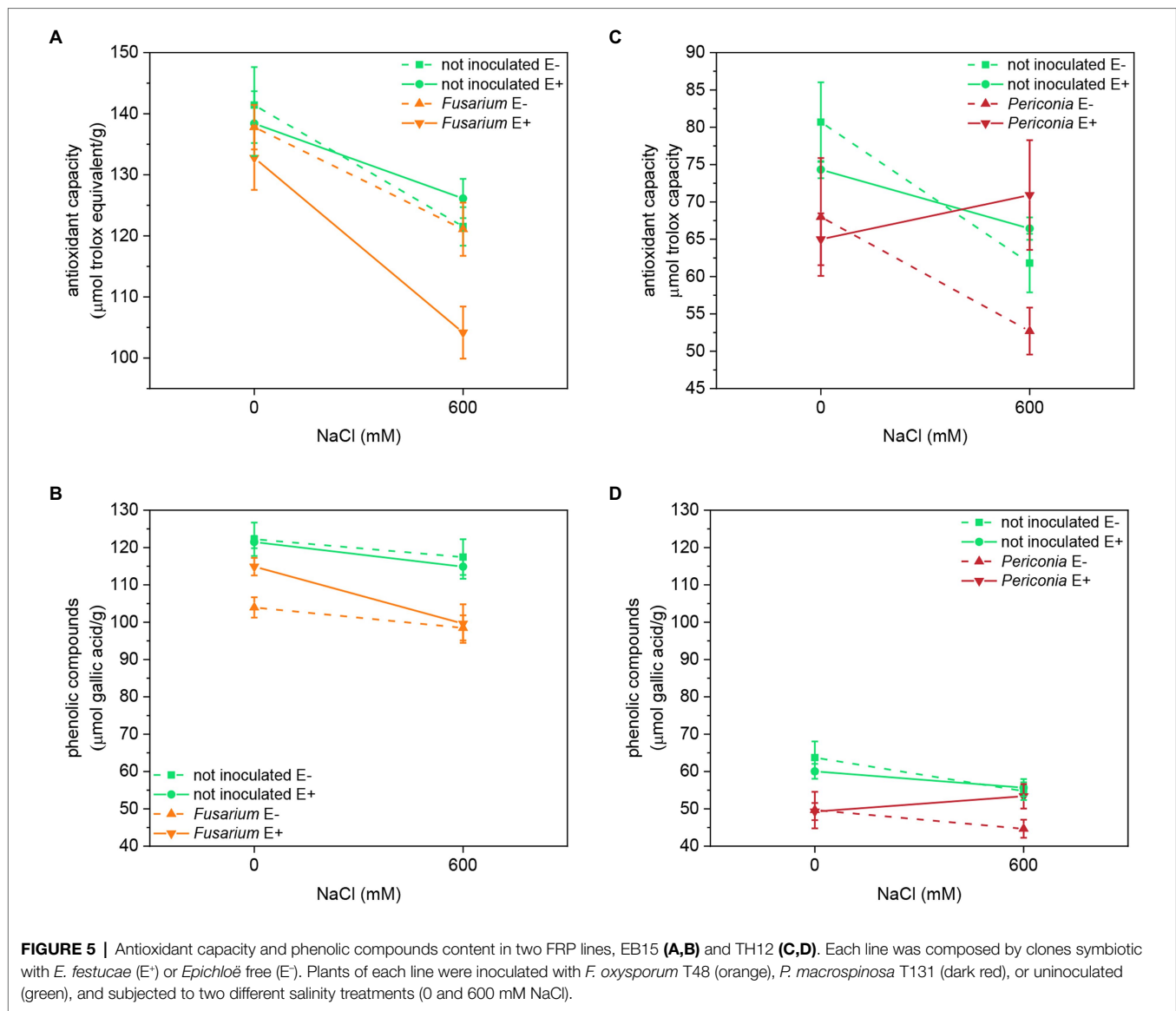
DISCUSSION

Plant Characteristics Related to Salinity Tolerance

The halophytic character of FRP was clearly observed in its response to salinity. The biomass reduction caused by exposure to high salinity was low, being statistically significant only for the leaf biomass of line EB15, while the root growth was not significantly affected in any case. Regarding this, shoot growth

is reported as more sensitive to salinity than root growth in *F. rubra*, as well as in other plant species (Baumeister and Merten, 1981; Munns and Tester, 2008).

The response of ion and solute accumulation to salinity stress gives an insight into some mechanisms of adaptation of FRP to its natural environment in sea cliffs. As previously reported for *F. rubra* subsp. *litoralis* (Rozema et al., 1978), exposure to salinity caused a substantial increment in the shoot content of Na⁺ and proline in FRP. This type of response occurs in plant species having tissue tolerance to Na⁺. In contrast to the alternative mechanism of Na⁺ exclusion from leaf blades, plants having tissue tolerance accumulate Na⁺ in their tissues by sequestering it into cell vacuoles. When this occurs, an osmotic adjustment of the cytoplasm is needed to maintain cell turgor, and this is achieved by the synthesis and accumulation of proline or other compatible solutes, as well as K⁺ (Flowers and Colmer, 2008; Munns and Tester, 2008; Zhao et al., 2020). This balance with proline, a common osmolyte among halophytic grasses (Slama et al., 2015), occurred in FRP regardless of the presence of leaf or root symbionts. A significant increase in K⁺ leaf concentration also occurred in response to salinity, independently of the symbiotic status of the plants. Different responses in terms of K⁺ content have been observed in different halophytes; some maintain the K⁺ concentration regardless of salinity, while others, including some maritime halophytes like FRP, show increased K⁺ content under salinity as a stress tolerance strategy (Flowers and Colmer, 2008; Assaha et al., 2017).

Both FRP lines differed in salinity tolerance. Salinity had a significant negative effect on the leaf biomass of line EB15, but not on TH12. At the same time, the Na⁺ content of line TH12 was lower than that of EB15. A similar inverse relation

FIGURE 5 | Antioxidant capacity and phenolic compounds content in two FRP lines, EB15 **(A,B)** and TH12 **(C,D)**. Each line was composed by clones symbiotic with *E. festucae* (E+) or *Epichloë* free (E−). Plants of each line were inoculated with *F. oxysporum* T48 (orange), *P. macrospinosa* T131 (dark red), or uninoculated (green), and subjected to two different salinity treatments (0 and 600 mM NaCl).
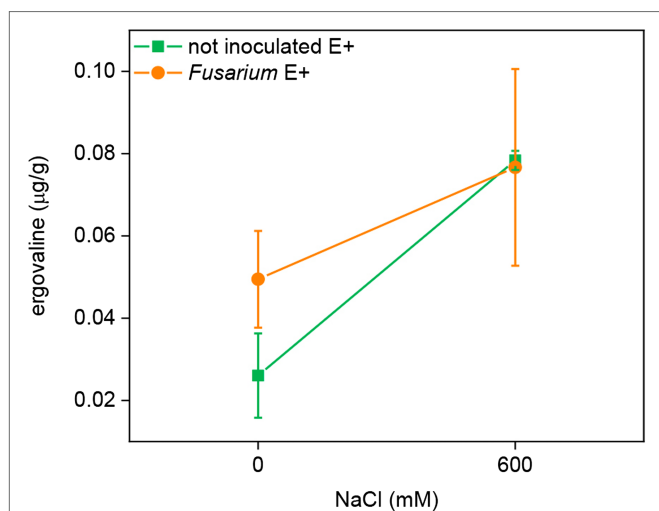
between salinity tolerance and Na+ content was reported to occur among subspecies of *F. rubra*, with the most tolerant subspecies having less Na+ content than the least tolerant inland subspecies (Rozema et al., 1978). Tolerance to salinity is an inheritable character in *F. rubra* (Humphreys, 1982), and the mechanisms related to the management of cellular Na+ are very likely to be related to it.

Epichloë Effects on Plants Subject to Salinity

Because of salinity, scarcity of soil and nutrients, and frequent wind exposure, the FRP habitat in sea cliffs could be considered suboptimal for plant growth. Considering that the incidence of *E. festucae* is relatively high in FRP populations, about 66% (Pereira et al., 2019), it could be expected that in such demanding habitat the benefits from this symbiosis must be greater than

its costs for the plant in order to support such incidence rates in a vertically inherited symbiont.

While some works report a clearly beneficial effect of *Epichloë* endophytes on several parameters related to salt tolerance in grasses (Song et al., 2015; Wang et al., 2020), others indicate that beneficial effects might be dependent on the grass-endophyte genotypes (Sabzalian and Mirlohi, 2010). In terms of leaf or root biomass, our results do not support an obvious or unambiguous role of *Epichloë* itself in FRP salt tolerance, confirming the results of a previous experiment (Zabalgogeazcoa et al., 2006b). However, the accumulation of Na+ was significantly lower in leaves of E+ than in E− plants of both plant lines. Such reduction in Na+ accumulation under salinity might be advantageous for salinity tolerance and habitat adaptation. In fact, among subspecies of *F. rubra*, the tolerance to salinity appears to be inversely correlated to Na+ accumulation (Rozema et al., 1978). A similar effect

FIGURE 6 | Ergovaline concentration in leaves of FRP line EB15 symbiotic with *E. festucae* (E⁺), inoculated with *F. oxysporum* T48 (orange) or uninoculated (green), and subjected to two different salinity treatments (0 and 600 mM NaCl).

of Na⁺ reduction in plants symbiotic with *Epichloë* was also reported in other grass species (Sabzalian and Mirlohi, 2010; Song et al., 2015). Nevertheless, other important fitness parameters, such as seed production or germination, have not been examined in relation to the *Epichloë* status of plants, and such information could help to understand the high prevalence of this symbiosis in sea cliff populations.

Plant protection against herbivores mediated by fungal alkaloids like ergovaline is often cited as a main driver of *Epichloë*-grass symbioses (Schardl et al., 2013). However, macroherbivores are absent from sea cliffs, and herbivore protection mediated by fungal alkaloids is not a satisfactory hypothesis to understand why *Epichloë* infection rates are high in sea cliff populations. It is worth noting that the incidence of *Epichloë* in *F. rubra* populations from sea cliffs is very similar to that observed in inland populations from semiarid grasslands (Zabalgogeazcoa et al., 1999; Pereira et al., 2019). Perhaps, other unknown factors than herbivory are as important for the favorable selection of these symbiotic associations.

Only plants of the line EB15 contained the fungal alkaloid ergovaline. Phenotypic variation in ergovaline content occurs among FRP plants, and in a previous survey, 21% of the plants analyzed did not contain this alkaloid (Vázquez de Aldana et al., 2007). Lack of ergovaline in symbiotic plants could be due to the absence of functional genes in the biosynthetic pathway, plant-fungal compatibility, environmental effects, or both (Schardl et al., 2013; Vázquez de Aldana et al., 2020b). The ergovaline content increased greatly in EB15 plants subject to salinity. Increased content of diverse alkaloids in response to salinity has been reported in several plant species (Wang et al., 2010; Li et al., 2020), including some ergot alkaloids produced by *Epichloë* in the grass *Achnatherum inebrians* (Zhang et al., 2011). Whether *Epichloë* alkaloids could have a function other than defensive in plants is unknown.

The antioxidant capacity of FRP plants under salinity stress did not differ in response to *Epichloë* symbiosis itself (in the absence of root endophytes). This result contrasts with those of Chen et al. (2018), who observed an increment in total antioxidant activity in E⁺ plants of *Hordeum brevisubulatum* subject to salinity. The fact that the functional intensity of the antioxidant machinery is time dependent, and several enzymes peak soon after stress exposure, but later return to a basal level (Baltruschat et al., 2008), might explain this difference. Alternatively, FRP is a halophytic marine plant species and has an efficient endogenous machinery to cope with salinity, where accumulation of antioxidants might not be responsible for the salt stress adaptation.
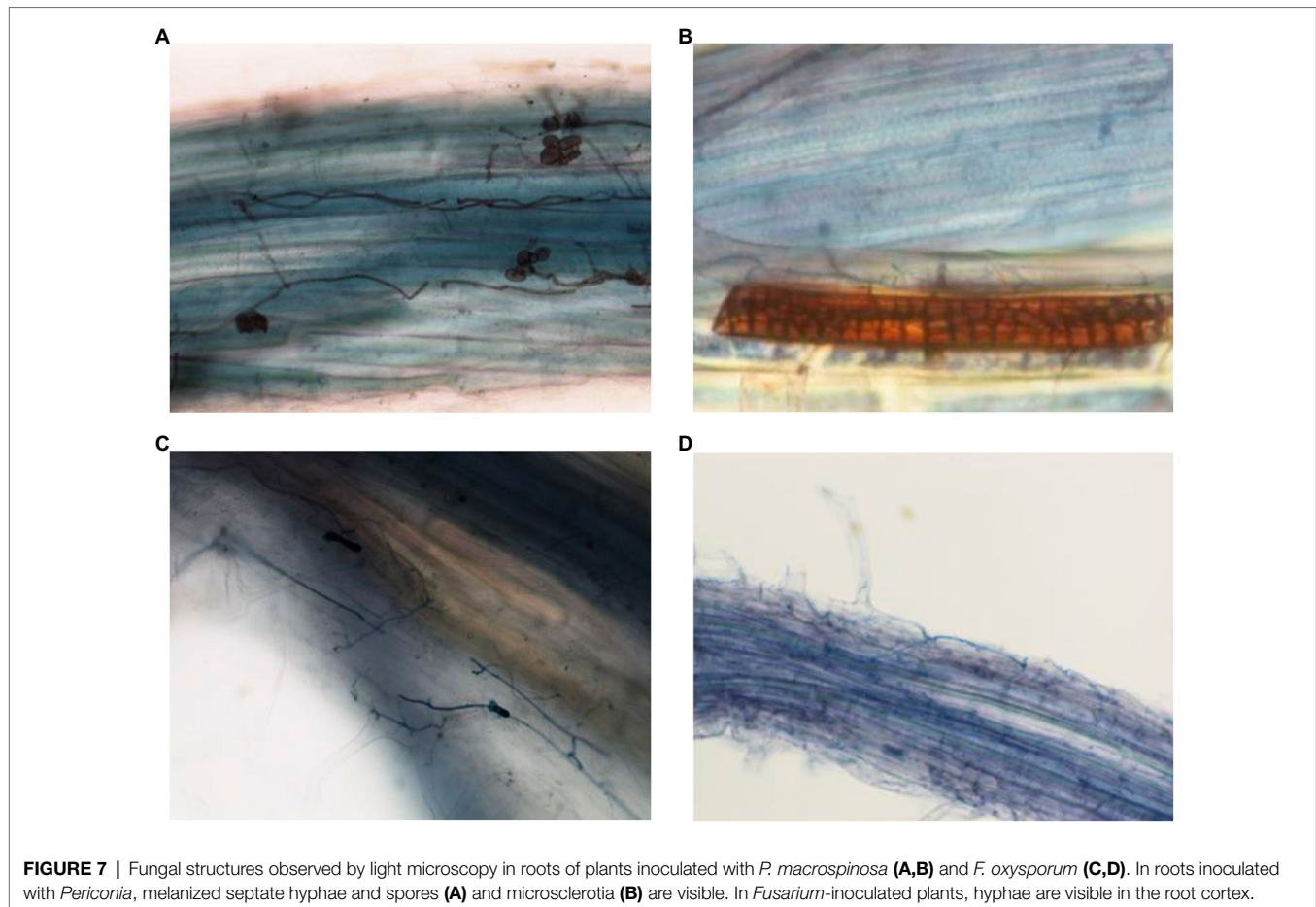
Root Endophyte Effect on Plants Subject to Salinity

The most remarkable results from this study are the observation that both *F. oxysporum* and *P. macrospinosa*, prevalent root endophytes in natural populations of FRP, have a significant beneficial effect on plant growth, regardless of the salinity treatment. Thus, these two fungal components of the FRP microbiome could favor plant hosts in their native habitat. *Fusarium oxysporum* is the most abundant fungal species detected in the root endosphere of FRP, with a prevalence of 57% in natural populations, and is a likely component of the core microbiome of this grass (Pereira et al., 2019). Asymptomatic root infections like the ones we observed in FRP plants are common for many *Fusarium* species (Bacon and Yates, 2006). *Fusarium oxysporum* is best known for its pathogenic *formae speciales* but also contains numerous strains having an endophytic lifestyle, like those present in FRP roots (Demers et al., 2015; Edel-Hermann and Lecomte, 2019). Endophytic, non-pathogenic, *F. oxysporum* strains may act as biocontrol agents against several root pathogens, including pathogenic strains of their own species (Constatin et al., 2020; de Lamo and Takken, 2020), and in some circumstances might promote plant growth in the absence of disease (Bitas et al., 2015).

Root endophytes, like *Serendipita indica* or *Fusarium culmorum*, have been reported to increase host plant tolerance to salinity (Waller et al., 2005; Rodriguez et al., 2008). The latter is a dominant endophyte in several organs of the beach grass *Leymus mollis*, and probably, it is an important microbiome component for the adaptation of the plants to their maritime habitat (Rodriguez et al., 2008). In view of our results, a similar function could be expected from *F. oxysporum* endophytes in FRP plants.

A parameter having a remarkable response in FRP plants inoculated with *F. oxysporum* was the Na⁺ content of leaves, which showed a pronounced decrease when compared to uninoculated plants. This response did not occur in plants inoculated with *P. macrospinosa*. As explained before, the results from our experiment suggest that in the absence of *F. oxysporum*, FRP plants cope with salinity by means of a tissue tolerance mechanism consisting of the vacuolar sequestration of Na⁺. However, plants also have other mechanisms to cope with salinity, like the exclusion of Na⁺ from entering the plants, a

FIGURE 7 | Fungal structures observed by light microscopy in roots of plants inoculated with *P. macrospinosa* **(A,B)** and *F. oxysporum* **(C,D)**. In roots inoculated with *Periconia*, melanized septate hyphae and spores **(A)** and microsclerotia **(B)** are visible. In *Fusarium*-inoculated plants, hyphae are visible in the root cortex.

mechanism centered in plant roots (Munns and Tester, 2008; Zhao et al., 2020). Thus, the association with *F. oxysporum* could be limiting Na$^+$ uptake from plant roots by means of modulating or complementing the plant Na$^+$ exclusion machinery. A similar situation of symbiont-mediated Na$^+$ exclusion has been reported in *Arabidopsis* inoculated with *S. indica* (Abdelaziz et al., 2019; Lanza et al., 2019).
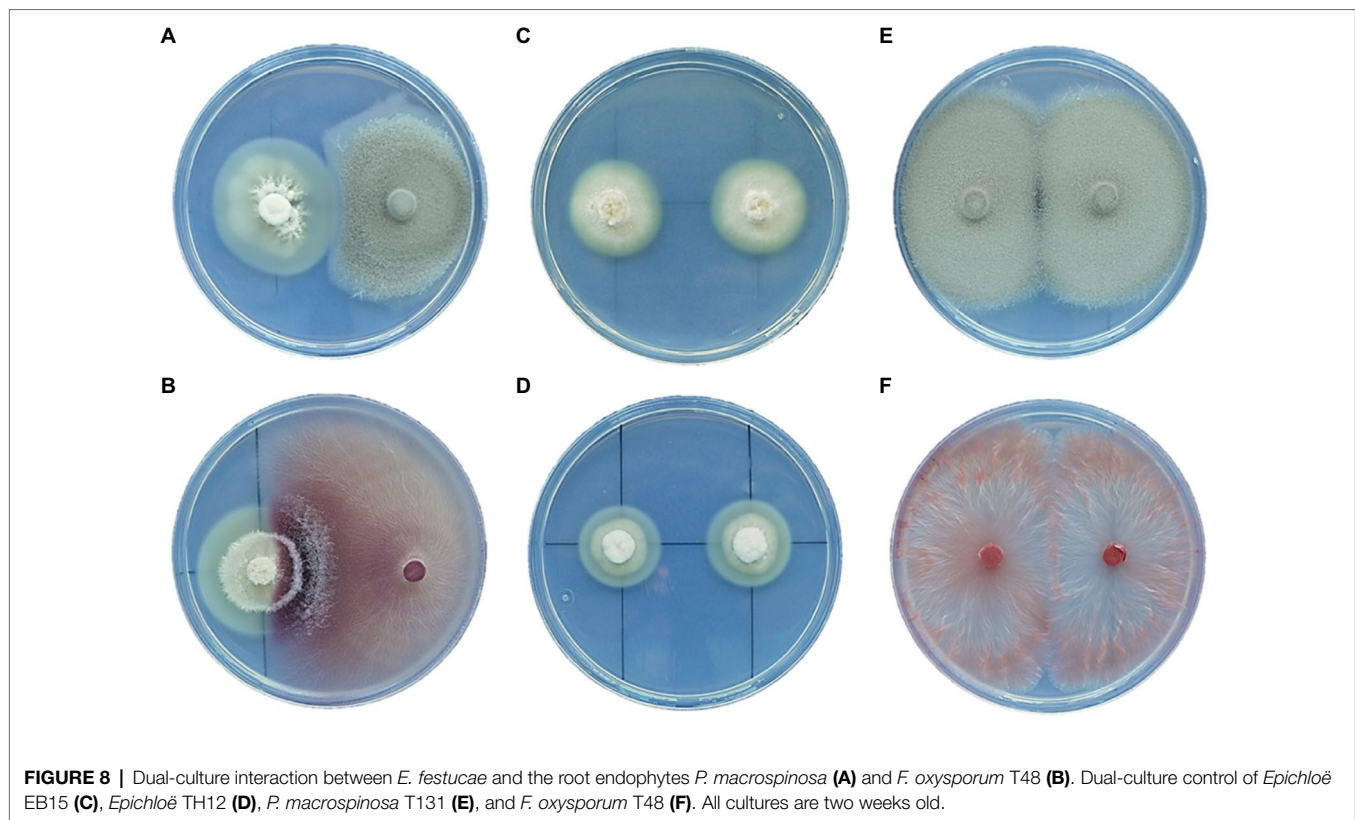
In addition, the growth promotion observed in the absence of salt in plants inoculated with *F. oxysporum* might be related to nutrient acquisition, an important issue in a habitat where soil is scarce or absent. Improved nutrient acquisition mediated by symbiotic fungi could occur in several ways; for instance, root endophytes could help to recycle dead plant material (Upson et al., 2009; Vázquez de Aldana et al., 2013), produce plant hormones that stimulate root growth (Sirrenberg et al., 2007), or alter the chemistry or microbiota of the rhizosphere (Alegría Terrazas et al., 2016). Thus, the increased shoot biomass observed in inoculated plants could be due to the greater root size caused by hormonal stimulation, increased nutrient availability, or both together mediated by *F. oxysporum*.

Periconia macrospinosa is a DSE with a wide host range, which has been described in roots of numerous grasses and other plants (Mandyam et al., 2012). In natural populations of FRP, the incidence of this taxon was about 16%, being one of its most abundant root endophytes (Pereira et al., 2019).

The search for the ecological function of DSE symbioses has been elusive, although some studies report increased biomass and nutrient (N, P) content, as well as salt tolerance in host plants (Mandyam and Jumpponen, 2005; Newsham, 2011; Gonzalez Mateu et al., 2020). In the present study, *P. macrospinosa* caused a significant increase in leaf and root biomass of its original host plant, FRP, in the presence as well as in the absence of salinity stress. *Periconia macrospinosa* is known to produce a wide range of extracellular enzymes able to metabolize numerous substrates, organic as well as inorganic (Mandyam et al., 2010; Knapp and Kovács, 2016). This fungus, which is thought to have a life cycle as a latent saprobe, could be involved in nutrient cycling and mobilization (Yakti et al., 2018). In the habitat of FRP plants, where soil is often nonexistent, nutrient cycling from dead plants or other organic remains could be very important for habitat adaptation. In contrast with *F. oxysporum*, this fungus did not reduce the Na$^+$ content of the plants; therefore, as a symbiont, its contribution might not be related to salinity tolerance.

Interactions Among Holobiont Components

Festuca rubra is a mainly outcrossing species that can also reproduce asexually by means of vegetative tillers produced from rhizomes (Harberd, 1961). Thus, populations of *F. rubra*

FIGURE 8 | Dual-culture interaction between *E. festucae* and the root endophytes *P. macrospinosa* **(A)** and *F. oxysporum* T48 **(B)**. Dual-culture control of *Epichloë* EB15 **(C)**, *Epichloë* TH12 **(D)**, *P. macrospinosa* T131 **(E)**, and *F. oxysporum* T48 **(F)**. All cultures are two weeks old.

could be conceived as groups of genotypically distinct individuals, some of which could be more or less represented by means of clonal expansion. As we found in this study, distinct plant genotypes can differ in their tolerance to salinity, an important trait for adaptation to sea cliffs. *Epichloë festucae* endophytes interact with plant genotypes in *Festuca* populations. As observed, the response to salinity of the plant individuals can be modified by their interaction with *Epichloë* endophytes, which reduce their Na$^+$ accumulation. Thus, the adaptation to salinity of both plant genotypes might be augmented by symbiosis with *Epichloë*. Further interactions seemed to occur with root endophytes. For instance, in terms of root growth and Na$^+$ accumulation, *P. macrospinosa* was positive for E$^-$, but not for E$^+$ plants, and the trend seemed to be opposite in the case of *F. oxysporum*. In addition, although *Epichloë* and root endophytes occupy different plant compartments, the dual-culture experiments suggested that *E. festucae* cultures respond to the presence of both root endophytes. In these experiments, the growth of *Epichloë* was stimulated by the presence of the root endophytes, and *Periconia* was inhibited by *Epichloë*, but *Fusarium* was not. Taking into account that more than 100 species of culturable fungi have been identified in FRP roots (Pereira et al., 2019), multiple interactions among microbiome components are likely to have important effects on distinct plant genotypes. Such a complex landscape of interactions affecting holobiont performance might help to understand why FRP populations having a 100% incidence of *E. festucae* are rare in marine and inland ecosystems. Even if *E. festucae*

is beneficial for some symbiotic plants, other holobiont configurations might compensate for its absence.

CONCLUSION

This study sheds light on how a plant supported by its microbiome can adapt to an inhospitable habitat in sea cliffs. To cope with salinity, FRP seems to rely on a tissue tolerance mechanism that allows its cells to accumulate Na$^+$, possibly in vacuoles, and an osmotic counterbalance occurs in the cytoplasm by means of proline and K$^+$. In addition to these intrinsic plant mechanisms, *E. festucae*, *F. oxysporum*, and *P. macrospinosa*, three fungal endophytes highly prevalent in natural populations of *F. rubra* and also contributed to improve plant performance under salinity. *Epichloë* caused a Na$^+$ reduction in leaves under salinity, which might be associated with salinity tolerance and plant survival in the long term. *Fusarium oxysporum*, the most abundant root endophyte from *F. rubra*, appears to contribute two different adaptive functions to symbiotic plants: first, promotion of the growth of leaves and roots in the presence as well as in the absence of salinity, and second, it caused a decrease in leaf Na$^+$ content under salinity, a function which as above suggested for *Epichloë* could improve plant adaptation to salinity. *Periconia macrospinosa* promoted the growth of leaves and roots of *F. rubra* plants regardless of the salinity treatment. Although the mechanisms mediating growth promotion or salinity tolerance are unknown, each of these

three components of the *F. rubra* core mycobiome contributed different functions, which are beneficial for plant adaptation to its habitat in sea cliffs, supporting our initial hypothesis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

EP designed and made the experiments, performed the chemical analyses, root microscopy, and dual-culture assays, and analyzed the data. All authors worked in the design of experiments, analysis of data, and writing of the manuscript.

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