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

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Festuca rubra subsp. pruinosa is a perennial grass that inhabits sea cliffs of the Atlantic coasts of Europe. In this inhospitable 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 inhospitable 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 (Humphreys, 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; Zabalgozaka 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 has been reported to occur in some characteristics. 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 temperature was 11.06 and 10.62 ℃, and the average annual temperature 13.5 and 13.8℃ 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.
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.

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).

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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 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 cellulase activity (Hankin and Anagnostakis, 1975).

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 populations the number of fungal taxa ranged from 34 to 59 (Table 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 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).

**RESULTS**

### 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 | 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.
or more populations: *Fusarium oxysporum* (57.1%), *Diaportha* 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.

### 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    | *Diaportha* 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.
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+ (*Slopeiomycyes 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 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).

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 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.

Effect of FRP Endophytes on Growth of *Lolium perenne*

A two-way ANOVA showed a significant effect of salinity ($p = 0.004$; $X_{\text{control}} = 0.236$ g, $X_{\text{NaCl}} = 0.192$ g), endophyte inoculated ($p < 0.001$; $X_{\text{control}} = 0.194$ g, $X_{\text{Periconia}} = 0.231$ g, $X_{\text{Penicillium}} = 0.109$, $X_{\text{Diaporthe}} = 0.321$ 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
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 mycoflora 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., *Sloeomyces clyndrosorus* 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; Löfgren 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* *E*. *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 macrospinoa*, *Sloeomyces 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 Junpponen, 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 inhospitable 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 mycorrhizal 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 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        | 0.63 | 1.000 | 0.300 | 0.182 | 0.154 |
| EDB        | 0.58 | 0.70 | 1.000 | 0.193 | 0.182 |
| SPR        | 0.84 | 0.88 | 0.88 | 1.000 | 0.238 |
| CDP        | 0.68 | 0.78 | 0.77 | 0.84 | 1.000 |
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 *Periconia 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 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.

**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 (Zabalgozacoa et al., 2006). The relatively high incidence of *E. festucae* suggests that in an inhospitable 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%) (Zabalgozacoa 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 Kotonen, 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 arundinaceus*) (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 F. 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   | Darkidea sp.                 | +                  | −                |
| C7   | Darkidea 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             | −                  | −                |
| C1   | Darksidea sp.                | +                  | −                |
| C7   | Darksidea sp.                | +                  | −                |
| S11  | Diaporthe sp. A             | −                  | −                |
| C2   | Darksidea sp.                | +                  | −                |
| C7   | Darksidea sp.                | +                  | −                |
| 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. (+++) High activity; halo > 5 mm.

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.

FIGURE 7 | Effect of inoculation with strains *Periconia* S6, *Penicillium* E7 and *Diaporthe* S69, isolated from F. 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).
The effect of (Ponce et al., 2009; Vázquez de Aldana et al., 2011). The presence of *Epichloë* did not alter fungal colonization in roots (Vandegrift et al., 2015; Slaughter and McCulley, 2016) or shoots (Zabalgoaeeaza 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 cylinosporus*, 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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**AUTHOR CONTRIBUTIONS**

EP collected the plants, isolated and identified fungi, made the experiments, and analyzed the data. BA designed the experiments, participated in plant collection, and analyzed the data. LS made the statistical analyses. IZ supervised the research, helped to sample plants, designed the experiments and analyzed the data. EP, BA, and IZ wrote the article.

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**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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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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