



# Disruption of Traditional Grazing and Fire Regimes Shape the Fungal Endophyte Assemblages of the Tall-Grass *Brachypodium rupestre*

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The plant microbiome is likely to play a key role in the resilience of communities to the global climate change. This research analyses the culturable fungal mycobiota of *Brachypodium rupestre* across a sharp gradient of disturbance caused by an intense, anthropogenic fire regime. This factor has dramatic consequences for the community composition and diversity of high-altitude grasslands in the Pyrenees. Plants were sampled at six sites, and the fungal assemblages of shoots, rhizomes, and roots were characterized by culture-dependent techniques. Compared to other co-occurring grasses, *B. rupestre* hosted a poorer mycobiome which consisted of many rare species and a few core species that differed between aerial and belowground tissues. Recurrent burnings did not affect the diversity of the endophyte assemblages, but the percentages of infection of two core species -*Omnidemptus graminis* and *Lachnum* sp. -increased significantly. The patterns observed might be explained by (1) the capacity to survive in belowground tissues during winter and rapidly spread to the shoots when the grass starts its spring growth (*O. graminis*), and (2) the location in belowground tissues and its resistance to stress (*Lachnum* sp.). Future work should address whether the enhanced taxa have a role in the expansive success of *B. rupestre* in these anthropized environments.

**Keywords:** *Brachypodium rupestre*, disturbances, fire recurrence, culturable endophytes, *Omnidemptus graminis*, *Lachnum* sp., *Epichloë typhina*

## INTRODUCTION

The study of the plant microbiome is a powerful tool that contributes to the understanding and prediction of the resilience of plant communities to global change (Vandenkoornhuys et al., 2015). In extreme environments, where ecosystems are fragile and very sensitive to changes, the plant microbiota is crucial to understand the adaptive capacity of plant communities. Low temperatures, soil acidity, low rates of mineralization, and low nutrient availability are some of the common traits of arctic and alpine ecosystems, where the study of the composition and function of the plant-microbial consortium has been addressed in the last decades (Li et al., 2012; Poosakkannu et al., 2014; Rudgers et al., 2014; Bråthen et al., 2015; Kotlínek et al., 2017).

Current research reports that for a particular ecosystem, the composition and diversity of the fungal microbiome of a plant host depends on its potential for carbon provision (plant abundance and biomass), on the fungal propagule availability, and on environmental factors, mainly climate, rather than on the taxonomy of the plant host (Higgins et al., 2014; Ranelli et al., 2015; Glynou et al., 2016; Kivlin et al., 2019). The first factor, potential for carbon provision, gives support to the resource-diversity hypothesis, the highest plant carbon determines the highest guest diversity (Wang et al., 2020), and this effect is modulated by the richness and diversity of the neighboring host plants and their own fungal populations (Hiiesalu et al., 2017; Vannier et al., 2020). Regarding the climate, the historical and current precipitation regime is known to play a key role in fungal endophyte composition (Lau et al., 2013; Liu et al., 2017), and its consequences in a scenario of climate change are a growing subject of study in recent years (Giauque and Hawkes, 2016; Koide et al., 2017; Slaughter et al., 2018).

In addition to the climate change, plant communities in some cold environments are being affected by other significant mechanisms of change (Pauchard et al., 2009). Many high altitude grasslands undergo profound changes due to the disruption of the historical regime of disturbances that created and preserved them (Lasanta-Martínez et al., 2005; Komac et al., 2013). Fire and herbivory are two crucial disturbances shaping the landscape of natural grasslands worldwide (Archibald et al., 2005; Anderson and Hoffman, 2007). Extensive grazing by livestock in highlands has sharply declined in Europe in the last decades. Grazing is considered the major driver of plant guild composition and diversity in grassland communities (Milchunas et al., 1988; Canals and Sebastia, 2000; Frank, 2005; Eldridge and Delgado-Baquerizo, 2018). Grazing has profound effects on soils, promoting root exudation and carbon flow exchange to the rhizosphere (Dawson et al., 2000; Hamilton and Frank, 2001), enhancing soil microbial biomass and activating the soil biogeochemical cycles (Bardgett and Wardle, 2003; Shaw et al., 2016), increasing and redistributing nutrients availability (Augustine et al., 2003; Liu et al., 2017) and affecting soil aeration by compaction (Jing et al., 2014). Through these effects on plants and soils, grazing may affect the structure of the fungal microbiome of plants (Schulz and Boyle, 2005; Wang et al., 2020).

Regarding fire, some high-altitude areas are currently experiencing an intensified regime of anthropic fires to reduce shrub encroachment and biomass build-ups caused by the relaxation of grazing activities (Köhler et al., 2005; Múgica et al., 2018). The effects of prescribed fires on soil properties have been documented in a range of contrasted habitats (Alcañiz et al., 2018), including high-altitude grasslands (in the Pyrenees, Armas-Herrera et al., 2016; San Emeterio et al., 2016). Although prescribed burnings do not reach the high temperatures of wildfires, their short and mid-term effects (thermal shocking, aboveground plant combustion, ash deposition, nutrient mineralization...) may drive profound changes in the plant community composition if the fire regime increases in recurrence (Uys et al., 2004). Regarding microbial soil communities, even low-intensity fires may depress soil microbial biomass (Múgica et al., 2018), with complex

consequences on the C and N cycles (Soong and Cotrufo, 2015; Shaw et al., 2016; Pellegrini et al., 2020). The composition of soil fungal communities has been documented to be disrupted by fires as well (Artz et al., 2009; Egidio et al., 2016; Semenova-Nelsen et al., 2019). Few studies have addressed the effects of fire on plant fungal assemblages (Bellgard et al., 1994; Eom et al., 1999; Mataix-Solera et al., 2002), although changes in species richness have been detected in foliar endophytes of burned trees (Huang et al., 2016), and specific plant-fungal mutualisms and pyrophilous fungal species have been described (Baynes et al., 2012; Raudabaugh et al., 2020).

The western Pyrenees encompass large areas affected by the decoupling of traditional fire and grazing practices. As a result of decreasing herbivore pressure and increasing burnings, a native perennial grass is expanding. *Brachypodium rupestre* (Host) Roem. & Schult (Schippmann, 1991; Schippmann and Jarvis, 1988) (= *B. pinnatum* subsp. *rupestre* (Host) Schübl. & Martens according to some authors), (Aizpuru et al., 1999), dominates grasslands and causes a severe loss of sympatric species (Canals et al., 2014; Canals, 2019), a phenomenon also observed in other European mountain ranges (Buckland et al., 2001; Holub et al., 2012; Tardella et al., 2018). In this natural setting, many questions arise concerning the response of the plant holobiont to the changed disturbance regime. To what extent the composition of the *B. rupestre* mycobiome responds to the altered disturbance regime? Does the species harbor a specific fire-adapted mycobiome in frequently burnt areas? Or on the contrary, is there sufficient inertia to maintain a similar mycobiome in a common climatic environment (cold temperatures, high rainfall and humidity), independently of the current disturbance regime? Since taxon-specific endophytes (such as *Epichloë* sp.) may confer a higher adaptive advantage in stressful situations than broadly distributed non-systemic endophytes (Hill et al., 1989; Malinowski and Belesky, 2000; Pereira et al., 2019; Harrison and Griffin, 2020), is the mycobiome of the plant responding to these expectations?

The main objective of this research was to characterize the culturable fungal endophyte community of *B. rupestre* across a well-defined gradient of disturbance. The gradient encompassed sites with different burning recurrences and levels of herbivory, which have led to different patterns of grassland structure and diversity. We also studied the fungal endophyte community of the aerial tissues of the most frequent companion species of *B. rupestre* in grasslands, *Festuca rubra* and *Agrostis capillaris*. The purpose was to estimate whether the major diversity of coexisting plant species (and potential fungal propagules) in diverse grasslands had a positive influence in the fungal assemblage of *B. rupestre*.

## MATERIALS AND METHODS

### Study Site and Sampling Design

The study area was located in the Aezkoa Valley in the western Pyrenees (43°3' N 1°13' W; 800–1900 m.a.s.l.), and occupies an area of 198 Km<sup>2</sup>. Because of the influence of the Atlantic ocean (55 km away in a straight line) the weather is cold and snowy

during winter, and mild and foggy during summer. The mean annual temperature is 9.3°C and mean annual precipitation is 1856 mm, according to data collected during 1989–2019 at the nearest climatic station, Irabia at 822 m.a.s.l.<sup>1</sup>. Soils are mainly derived from sandstones and calcareous clays, acidic with high organic matter and loamy or clay-loamy textures. The landscape is a mosaic of beech forests, gorse shrublands dominated by *Ulex gallii* Planch and *Erica vagans* L., and grasslands dominated by perennial grasses such as *Festuca rubra* gr., *Agrostis capillaris* L., *Brachypodium rupestre* (Host) Roem. & Schult, *Danthonia decumbens* (L.) and *Avenula sulcata* (J. Gay ex Boiss) Dumort. Other species are *Galium saxatile* L., *Potentilla erecta* (L.) Ræusch, *Potentilla montana* Brot., *Hypochaeris radicata* L., and the legume *Trifolium repens* L. The area is part of the European Protected Areas Network (Natura 2000) and was declared Special Area of Conservation (Roncesvalles-Selva de Irati, code ES0000126) in 2011 (Figure 1).

High-altitude grasslands are extensively grazed from May to October by sheep, cows and horses. Domestic herbivores have been present in the area since the Neolithic, as numerous prehistoric pastoral remains indicate. Where an appropriate extensive summer grazing exists, grasslands constitute high-diversity communities that are burned every 6–7 years by the bush-to-bush traditional practice to control localized shrub resprouting (San Emeterio et al., 2016). However, in the last decades, the number of grazers has sharply declined due to socio-economical changes experienced in the valleys, and the use of repeated winter burnings to control the build-up of ungrazed biomass has increased (Durán et al., 2020). Nowadays, many

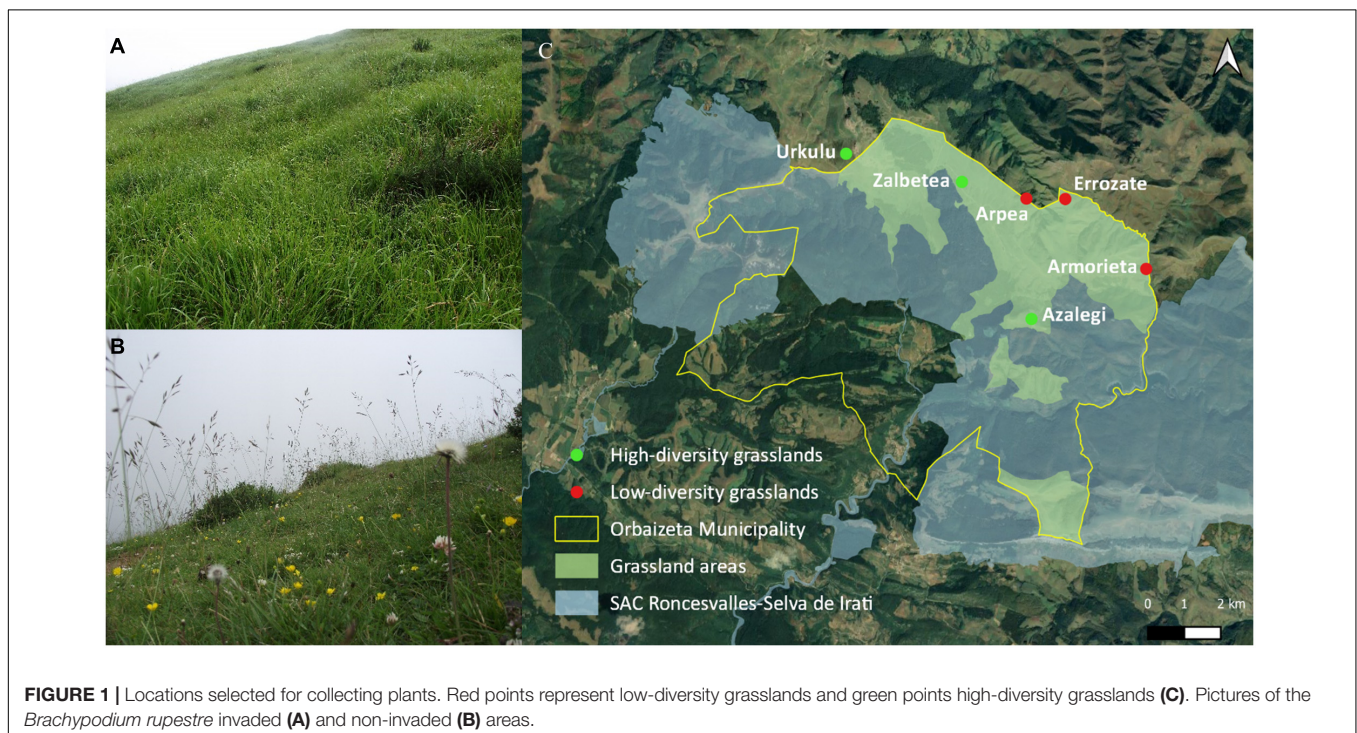
areas are burned recurrently, every 1–2 years (Canals et al., 2017). Due to the frequent burnings and the lack of grazing, the grasslands are far less diverse, favored by the expansion and dominance of *B. rupestre*, which grows in dense and tall clumps. Consequently, the current open landscape is a combination of grassland communities with different degrees of cover by *B. rupestre*, which accurately reflects the level of herbivory and the burning recurrence.

Based on the cartographic information provided by the Management Plan of the area (Ferrer and Canals, 2008), we selected six grassland communities where *B. rupestre* was present at different coverages: three areas had above 75% cover and three areas under 25% cover (Table 1 and Figure 1). The detailed floristic communities of the area are available in Durán et al., 2020. At each location, 40 turfs (400 cm<sup>3</sup>) of *B. rupestre* (including shoots, rhizomes, roots and rhizosphere) were collected in summer 2018. We established a distance of ca 30 m among collected plants to avoid clonal individuals (Baba et al., 2012). In the three areas of diverse grasslands, 40 turfs of *Festuca rubra* and 40 turfs of *Agrostis capillaris* were also collected. Sampling points were georeferenced and the sampling grid covered ca 2.5 ha per location (Supplementary Figure 1). In total, 480 turfs were collected, placed in seedbed trays and transported to the UPNA laboratory. The plants in the turfs were processed for the isolation of fungi from shoots, rhizomes and roots in the same week.

## Isolation and Identification of Fungi

We isolated fungi from shoots, rhizomes and roots of *B. rupestre* and from shoots of *F. rubra* and *A. capillaris*. Leaf sheaths and stems were cut into fragments of ca 5 mm, and surface-disinfected by immersion in a solution of 20% commercial bleach (1% active

<sup>1</sup><http://meteo.navarra.es>



**TABLE 1** | Characteristics of the six locations selected.

Location	Fire recurrence	Grazing level	<i>B. rupestre</i> cover (%)	Plant diversity
1 Arpea	High	Low to non-existent	>75%	Very low
2 Errozate	1–2 years			
3 Armorieta				
4 Urkulu	Low	Moderate	<25%	High
5 Zalbetaea	6–7 years			
6 Azalegi				

chlorine) containing 0.02% Tween 80 (v:v) for 10 min, and finally rinsed with sterile water. The fragments of rhizomes and roots were surface-sterilized with the same bleach-Tween 80 solution, but then treated with an aqueous solution of 70% ethanol for 30 s, and a final rinse with sterile water. About 10–12 tissue fragments of the same individual were plated in a Petri dish with potato dextrose agar (PDA) containing 200 mg/L of chloramphenicol to avoid the growth of endophytic bacteria. Petri dishes, kept at room temperature and ambient light, were checked daily for mycelium growth during 5 weeks. When mycelium emerged from a tissue fragment, a small amount was transferred to a new Petri dish to obtain a culture.

Isolated fungi were grouped into morphotypes according to their morphological characteristics (colony color, exudates, growth type, and mycelium appearance). One or more isolates of each morphotype were genotyped for taxonomic purposes. To do this, a small amount of mycelium was scratched from the isolate culture and its DNA extracted using the Phire Plant Direct PCR Kit (Thermo Fisher Scientific). The ITS1, 5.8s and ITS2 regions were amplified using ITS4 and ITS5 primers (White et al., 1990). PCR amplification was done at 98°C for 5 min, followed by 35 cycles of 95°C for 5 s, 54°C for 5 s, 72°C for 20 s, and a final phase of 72°C for 1 min. Amplicons were purified (Favor Prep™ Plant Genomic DNA Extraction Mini Kit, Favorgen) and sequenced by the Sanger method at an external sequencing service (STABvida).

Because the range of intraspecific variation in ITS sequences is unknown for most fungal species (Taylor et al., 2000), DNA sequences were clustered using the CD\_HIT program (Li and Godzik, 2006; Huang et al., 2010) and those with 97% or more similarity were considered to belong to the same taxon. A representative sequence of each cluster was selected and used to search the database of the ITS region from fungi type and reference material (Schoch et al., 2014) at the National Center for Biotechnology Information (NCBI) using the BLAST algorithm. In addition, the UNITE database of fungal nucleotide sequences was used as a complement for sequences without type specimens in NCBI. We also used the database FUNGuild (Nguyen et al., 2016) to get information on the ecological guild of each taxon, and to estimate for their possible functional roles.

## Data Analyses

To evaluate the efficiency of our sampling effort for measuring species richness, species accumulation curves were estimated with all the species, and also excluding singletons, species that

appeared only once (Vegan package, Oksanen et al., 2017). We calculated the incidence of all endophyte species within each type of tissue (shoots, rhizomes and roots), grassland type (low and high-diversity) and host plant species, and determined richness and Shannon and Simpson diversity indexes (Vegan package, Oksanen et al., 2017). Differences in species richness and diversity indexes of *B. rupestre* endophytes were analyzed using two-way ANOVA with tissue and grassland type as fixed factors, and permutation tests was used to compare diversity indexes of *B. rupestre*, *F. rubra*, and *A. capillaris* shoots (Coin package, Hothorn et al., 2008).

Venn diagrams were used to represent the taxa shared among grassland types, plant tissues and grass species (Euler package, Chen and Boutros, 2011). Frequencies were calculated from the matrix of presence/absence of isolates and permutational analyses of variance (PERMANOVA) were used to evaluate the variability of the fungal endophyte assemblages of *B. rupestre* between grassland types and among plant tissues. For that purpose, Adonis function was used (Vegan package, Oksanen et al., 2017). Distances were calculated using Bray–Curtis dissimilarities, set the number of permutations to 9999 and constrained the permutations within each location. Since PERMANOVA analyses are very sensitive to heterogeneity of multivariate dispersions, homogeneous dispersion between treatment groups was tested using the betadisper function.

To identify which species were characteristic of a particular tissue and grassland type, the indicator species tests in the Labsdv package was used (Roberts, 2019). The indicator value of a species (indval) measures the fidelity and relative abundance of the species in a particular situation (Dufrêne and Legendre, 1997). General Linear Mixed Models (GLMM) was used to determine whether the indicator species for a particular grassland type presented differences in the probability of incidence among grassland types. The grassland type was included as the fixed factor, the location as the random factor, using a binomial distribution. In addition, GLMM was used to analyze whether the probability of incidence of some mycobiome core species from the aboveground tissues presented differences among host plant species. Host plant species was included as the fixed factor, location as the random factor, using a binomial distribution. GLMM's were done using the lme4 package (Bates et al., 2015).

## RESULTS

### Isolation and Identification of Fungal Endophytes

We plated ca 10000 tissue fragments of *B. rupestre* (shoots, rhizomes and roots), *F. rubra* (shoots) and *A. capillaris* (shoots) in 960 culture media plates (10–12 fragments per plate). We obtained 1151 isolates (ca 190 isolates per location) which were classified into a total of 95 morphotypes. One or more isolates of each morphotype were sequenced, obtaining 116 sequences. After the sequence clustering process, 61 different sequence types remained, which were identified into 53 different taxa using the NCBI and UNITE databases. Twenty-two of them were classified

to species rank, 20 to genus, 6 to family, 3 to order, and the remaining two to class rank (**Supplementary Table 1** contains the complete list of taxa identified and their accession number).

## Diversity of the *B. rupestre* Mycobiome

Fungi were isolated from 58% of the *B. rupestre* plants in low-diversity grasslands and from 43% in high-diversity grasslands (**Table 2**). Endophyte incidence varied among tissues: 37% of shoots, 52% of rhizomes and 64% of roots harbored fungi. Forty-five different taxa were identified in *B. rupestre*. Endophyte species richness in *B. rupestre* shoots, rhizomes and roots were 9, 25, and 26 taxa, respectively. Endophyte species richness in *B. rupestre* growing in low-diversity grasslands ranged from 9 to 22, and from 12 to 18 taxa in high-diversity grasslands (**Table 2**). Roots and rhizomes shared 23.9% of the species, whereas shoots with rhizomes and with roots shared 8.7 and 6.5%, respectively, all tissues had only 6.5% of all species in common (**Figure 2**). *B. rupestre* plants in low and high-diversity grasslands shared 39.1% of the species.

The most frequent species were *Albotricha* sp., *Lachnum* sp. B, *Omnidemtus graminis*, and Mollisiaceae sp. (**Table 3**). *Omnidemtus graminis* was more frequent in shoots than in rhizomes and absent in roots, whereas *Albotricha* sp., *Lachnum* sp. B, and Mollisiaceae sp. were more frequent in belowground tissues than in shoots. In addition to the Mollisiaceae sp. taxon, other dark septate endophytes (DSE) such as *Cadophora* sp., *Microdochium bolleyi*, *Microdochium neoqueenslandicum*, and *Periconia* sp. were found, although with a low incidence. Members of the *Clavicipitaceae* family, such as *Metapochonia bulbilosa*, *Metarhizium carneum*, and *Epichloe typhina* in shoots and rhizomes also occurred. The frequencies of the remaining species were under 2% (**Supplementary Table 2**). Three trophic types (pathotroph, saprotroph, and symbiotroph) were found in the mycobiome

of *B. rupestre*. The most common guilds were plant pathogens, endophytes and undefined saprotrophs. However, a remarkable number of taxa were animal pathogens (*Clonostachys rosea*, *Tolypocladium album*, and *Trichoderma koningii* are described as entomopathogens, *Metapochonia bulbilosa* and *Sarocladium strictum* as nematophagous). Fungi with antimicrobial activity (*Fusarium circinatum*, *Glarea* sp., *Nemania* sp., and *Penicillium ortum*) and species with a high source of bioactive compounds (*Acremonium* sp., *Gaeumannomyces* sp., and *Lachnum* sp.) also occurred (**Supplementary Table 1**).

The species-accumulation curves including all the endophyte taxa were non-asymptotic (**Figure 3**), but when singletons were excluded the curves reached a plateau, suggesting that an increase in the sampling effort would reveal few common taxa, and mostly rare species. Therefore, a sampling effort of 100 plants is adequate to flatten the curves of the different tissues when excluding the singletons (**Figures 3B–D**). A greater sampling effort would be needed to include all the tissues (**Figure 3A**), although low-diversity grasslands need more plants than high-diversity grasslands to characterize their endophyte richness (**Figures 3E,F**).

Species richness and diversity indexes were significantly different among tissues ( $F = 10.319$ ,  $p < 0.001$ ;  $F = 18.336$ ,  $p < 0.001$ ;  $F = 24.114$ ,  $p < 0.001$ ; for richness, Shannon and Simpson, respectively). Shoots showed consistently lower diversity values than rhizomes or roots in both types of grasslands (**Figure 4**). No significant differences in diversity were found between low and high-diversity grasslands ( $F = 0.210$ ,  $p > 0.05$ ;  $F = 0.180$ ,  $p > 0.05$ ;  $F = 0.311$ ,  $p > 0.05$ ; for richness, Shannon and Simpson, respectively).

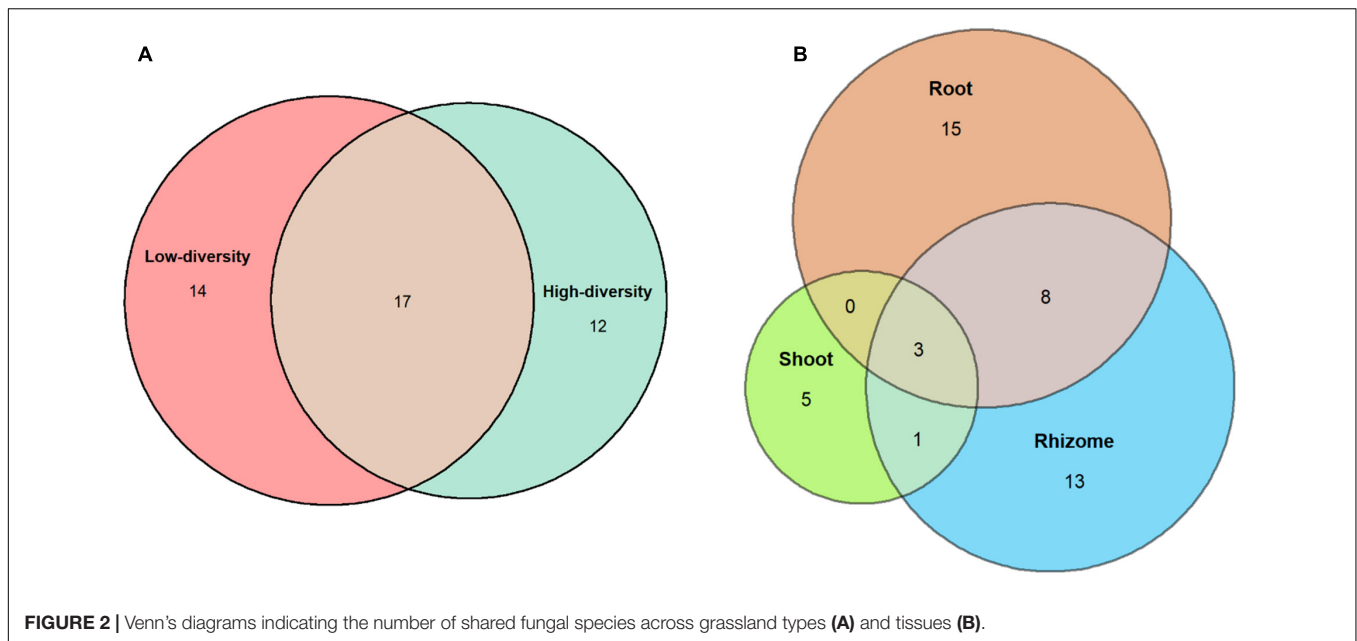
## Distribution Patterns of the *B. rupestre* Mycobiome

A homogeneous dispersion was found within different tissues (Beta-disper;  $F_{2,15} = 2.195$ ,  $p = 0.146$ ) and grassland types (Beta-disper;  $F_{1,16} = 0.028$ ,  $p = 0.870$ ), contrasting with the significant effects on community assemblages. PERMANOVA analysis showed that tissue and grassland type had a significant effect on the endophytic community of *B. rupestre* plants (both  $p < 0.001$ ). Tissue explained ca 50% of the variance of the model ( $r^2 = 0.508$ ), whereas grassland type explained ca 5% ( $r^2 = 0.052$ ). The NMDS (Non-Metric Multidimensional Scaling) plot discriminated the assemblages according to the tissues and included the indicator species (**Figure 5**). *Omnidemtus graminis* ( $p = 0.001$ ) and *Sarocladium strictum* ( $p = 0.032$ ) were indicators of shoots. *Metapochonia bulbilosa* ( $p = 0.003$ ), *Clonostachys rosea* ( $p = 0.011$ ), and *Tolypocladium album* ( $p = 0.009$ ) were indicators of rhizomes. And *Lachnum* sp. B ( $p = 0.001$ ), *Albotricha* sp. ( $p = 0.001$ ), Mollisiaceae sp. ( $p = 0.001$ ), *Lachnum* sp. A ( $p = 0.008$ ), *Acremonium* sp. ( $p = 0.021$ ), and *Dictyochoaeta* sp. ( $p = 0.024$ ) were indicators of roots. Regarding the grassland type, *Albotricha* sp. ( $p = 0.001$ ) was an indicator of high-diversity grasslands and *Lachnum* sp. B ( $p = 0.004$ ) and *Omnidemtus graminis* ( $p = 0.003$ ) of low-diversity grasslands.

GLMM's showed that *B. rupestre* plants from low-diversity grasslands had a greater probability of having their roots infected

**TABLE 2** | Number of plants collected, final plates (taking into account the contaminations), fungal endophyte incidence (% plants), and number of fungal species in *Brachypodium rupestre*, all of that according to tissue and location.

	Tissue	Number of plants	Number of plates	Endophyte incidence (% plants)	Number of fungal species
	Shoot	240	231	37	9
	Rhizome	240	213	52	25
	Root	240	228	64	26
	Total	240	672		45
Cover	Location				
Low-diversity grasslands	Arpea	40	107	59	21
	Errozate	40	112	60	9
	Armorieta	40	110	56	22
	Total	120	329		32
High-diversity grasslands	Urkulu	40	114	42	12
	Zalbeta	40	114	49	18
	Azalegi	40	115	39	16
	Total	120	343		30



**FIGURE 2** | Venn's diagrams indicating the number of shared fungal species across grassland types (A) and tissues (B).

by *Lachnum* sp. B (LRT = 4.5719,  $p = 0.032$ ) and their shoots by *Omnidemptus graminis* (LRT = 4.679,  $p = 0.030$ ) than plants from high-diversity grasslands (Figure 6). Regarding *Albotricha* sp., the patterns were not so clear and its probability of incidence in *B. rupestre* roots was not significantly different between grassland types (LRT = 0.2849,  $p = 0.594$ , Figure 6).

### Aboveground Endophyte Assemblages of the Most Common Grasses in High-Diversity Grasslands

The incidence of endophytes varied among the shoots of the three grass species, ranging from 25% in *B. rupestre* and *A. capillaris* to 38% in *F. rubra*. For each plant species, a strong variability of endophyte incidence among locations was detected (Table 4). Eight taxa not observed in the *B. rupestre* survey were isolated from *F. rubra* or *A. capillaris* (Supplementary Table 1).

A total of 23 taxa were isolated from the shoots of *B. rupestre*, *F. rubra* and *A. capillaris* (Supplementary Table 3). Endophyte species richness in *B. rupestre*, *F. rubra*, and *A. capillaris* shoots were 5, 10, and 16, respectively (Table 4). *F. rubra* and *A. capillaris* shared more endophyte species between them than *B. rupestre* with either (Figure 7). The three grasses had two endophyte species in common, *Lachnum* sp. B and *Omnidemptus graminis*. The most frequent endophyte species in *B. rupestre* shoots were *Omnidemptus graminis* and *Epichloë typhina*, in *F. rubra* *Epichloë festucae* and Mollisiaceae sp., and in *A. capillaris* *Lachnum* sp. B, *Epichloë baconii* and Mollisiaceae sp. (Table 5).

When including all endophyte species, the species-accumulation curves of the three host grasses were non-asymptotic (Figure 8), but when removing singletons the curves flattened with a sampling effort of 50 plants for *B. rupestre* and 60 plants for *F. rubra* and *A. capillaris* in high-diversity grasslands (Figure 8).

The mycobiome of *A. capillaris* appeared to be more rich and diverse (averaged per site richness<sub>AC</sub> = 7.0, Shannon<sub>AC</sub> = 1.85, Simpson<sub>AC</sub> = 0.83) than that of *F. rubra* and *B. rupestre* (averaged richness<sub>FR</sub> = 4.3, Shannon<sub>FR</sub> = 0.76, Simpson<sub>FR</sub> = 0.36; averaged richness<sub>BR</sub> = 2.7, Shannon<sub>BR</sub> = 0.49, Simpson<sub>BR</sub> = 0.26). However, the high variability among locations and the size of the sample (Figure 9) did not allow to detect significant differences in richness and diversity indexes among grasses in the permutations tests (richness,  $t = 1.7876$ ,  $p = 0.1737$ ; Shannon,  $t = 2.0697$ ,  $p = 0.09614$ ; Simpson,  $t = 2.0193$ ,  $p = 0.1076$ ).

*Omnidemptus graminis* and *Epichloë typhina* were species indicators of *B. rupestre* ( $p = 0.001$ ;  $p = 0.008$ ), whereas *Epichloë festucae* was a species indicator of *F. rubra* ( $p = 0.001$ ) and *Epichloë baconii* of *A. capillaris* ( $p = 0.009$ ). The GLMM showed that the probability of incidence of *Omnidemptus graminis* significantly varied among species (LRT = 38.194,  $p < 0.001$ ), and had a higher probability of incidence in *B. rupestre*, despite its presence in the other grasses (Figure 10). On the contrary, *Epichloë* species were specific of each grass (*E. typhina* in *B. rupestre*, *E. festucae* in *F. rubra* and *E. baconii* in *A. capillaris*), but the probability of *Epichloë* infection was higher in *F. rubra* than in the rest of grasses (LRT = 34.581,  $p < 0.001$ ; Figure 10).

## DISCUSSION

### Shoot Fungal Endophyte Assemblages of the Most Common Grasses

The richness and diversity of the mycobiomes from this survey was relatively low compared to other studies in grasses (Sánchez-Márquez et al., 2008, 2010; Pereira et al., 2019). The study of fungal endophytes is highly method-dependent (Hyde and Soyong, 2008). In this research, we applied a conservative

**TABLE 3 |** Incidence in *Brachypodium rupestre* plants of the most abundant fungal endophyte taxa, differentiated by tissues and number of locations where they were present.

Tissue	Fungal endophyte	Low-diversity grasslands		High-diversity grasslands	
		Incidence (%)	Number of locations	Incidence (%)	Number of locations
Shoots	<i>Omnidemptus graminis</i>	46.1	3	23.3	3
	<i>Epichloë typhina</i>	2.6	1	3.4	2
	<i>Sarocladium strictum</i>	3.5	2	0	0
Rhizomes	<i>Lachnum</i> sp. B	13.9	3	8.9	2
	Mollisiaceae sp.	14.9	2	7.1	2
	<i>Albotricha</i> sp.	2.0	1	16.1	2
	<i>Omnidemptus graminis</i>	5.9	1	5.4	2
	<i>Metapochonia bulbilosa</i>	3.0	1	2.7	3
	<i>Fusarium circinatum</i>	3.0	3	1.8	1
	<i>Penicillium ortum</i>	2.0	2	2.7	1
	<i>Tolypocladium album</i>	3.0	1	0.9	1
	<i>Clonostachys rosea</i>	2.0	1	1.8	2
	<i>Ilyonectria robusta</i>	2.0	2	0.9	1
	<i>Microdochium bolleyi</i>	0	0	2.7	2
	Roots	<i>Lachnum</i> sp. B	35.4	3	16.5
Mollisiaceae sp.		14.2	3	14.8	3
<i>Albotricha</i> sp.		9.7	3	19.1	2
<i>Lachnum</i> sp. A		3.5	3	2.6	2
<i>Ilyonectria robusta</i>		0.9	1	5.2	2
<i>Glarea</i> sp.		1.8	2	3.5	2
<i>Dictyochaeta</i> sp.		3.5	2	0	0
<i>Acremonium</i> sp.		1.8	2	1.7	1
<i>Fusarium circinatum</i>		0	0	3.5	2
<i>Mollisia</i> sp.		2.7	1	0	0

protocol, with along sterilization process -i.e., 10 min vs. 6 min in Pereira et al. (2019)-, and discarding the mycelia growing in the first day, which might influence the final counts of species (Burgdorf et al., 2014).

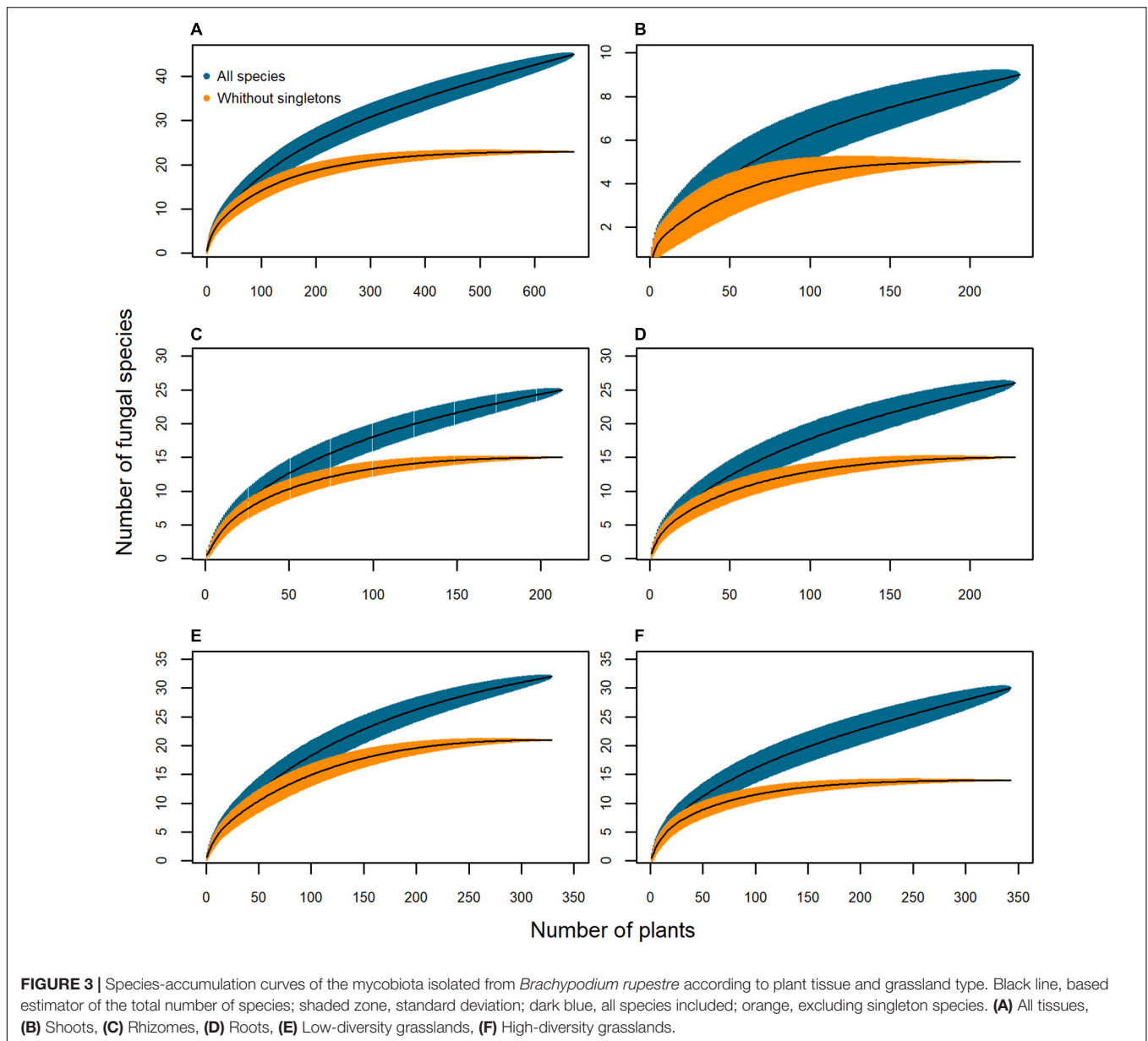
Despite the low richness reported, interesting patterns emerged from the shoot assemblages of the grasses. *B. rupestre* hosted the poorest mycobiome, half of that present in *F. rubra*, and one third of that of *A. capillaris*. Only two of 23 endophyte species identified were common to all grasses (*Omnidemptus graminis* and *Lachnum* sp. B). Recent studies indicate that plant

identity influences foliar fungal assemblages more than expected from previous literature (Kivlin et al., 2019). Since the three grasses are sympatric and share an identical abiotic environment (i.e., climate, soil, location), other factors might define their mycobiomes. Plant abundance and capacity for carbon provision (*B. rupestre* develops more biomass but it is generally less abundant in diverse grasslands than the other grasses), potential interactions among fungal species (i.e., *Omnidemptus graminis*, which is abundant in *B. rupestre*, can induce host defense against other fungi (Schulz and Boyle, 2005; Constantin et al., 2020) and the different degree of herbivory (*B. rupestre* is less palatable to livestock than *A. capillaris* and *F. rubra*), may play a definite role in the structure of fungal assemblages.

Regarding systemic endophytes, the three grasses were infected by species of the genus *Epichloë*. *E. typhina*, *E. baconii*, and *E. festucae* are highly specific for *B. rupestre*, *A. capillaris*, and *F. rubra*, respectively (Leuchtmann et al., 2014; Saikkonen et al., 2016). *Epichloë* species are known to produce long-term, systemic infections and have a narrow range of hosts, limited to a genus or to related genera (Schardl et al., 2009; Schirrmann et al., 2015). In the area of study, the incidence of *Epichloë* was variable depending on species and locations, and symbioses with *E. festucae* were by far the most abundant (up to 50% incidence in one location) compared to *E. typhina* and *E. baconii* (less than 8% incidence in all locations). This is in accordance with previous research showing that the incidence of *Epichloë*-grass infections change depending on the associations (Leuchtmann and Schardl, 1998). *E. festucae* mainly reproduces asexually by vertical transmission to seeds, while in *E. typhina* and *E. baconii* seed transmission is absent (Leuchtmann et al., 2014). *B. sylvaticum*, a species close to *B. rupestre*, is very common in forest undergrowth, and displays high levels of infection by *E. sylvatica*, a seed-transmitted species (Meijer and Leuchtmann, 1999; Brem and Leuchtmann, 2001). The results suggest that the capacity of systemic endophytes to infect host seeds and transmit vertically, which is the most common reproductive mechanism in *Epichloë* species, leads to higher levels of incidence than in sexual, horizontally transmitted *Epichloë* species. This makes sense because in vertically transmitted endophytes reproductive fitness is intimately tied to that of their hosts (Saikkonen et al., 2002). As a consequence, mutualisms are expected to be more successful and intense in these situations. Of the three *Epichloë* species identified, *E. festucae* has been extensively studied for its capacity to establish successful symbioses in the most extreme environments (Vázquez-de-Aldana et al., 2013b; Zabalgozcoa et al., 2013; Leinonen et al., 2019; Pereira et al., 2019), and for the variety of advantages that confers to the host plant (i.e., tolerance to stress, resistance to herbivores, plant fitness) (Malinowski and Belesky, 2000; Zaurov et al., 2001).

## Fungal Tissue Assemblages and Core Mycobiome of *B. rupestre*

Roots and rhizomes of *B. rupestre* displayed different endophyte assemblages than aboveground tissues, had the greatest percentages of fungal infection (Figure 5), and a significantly high richness and diversity of taxa. Previous studies have shown

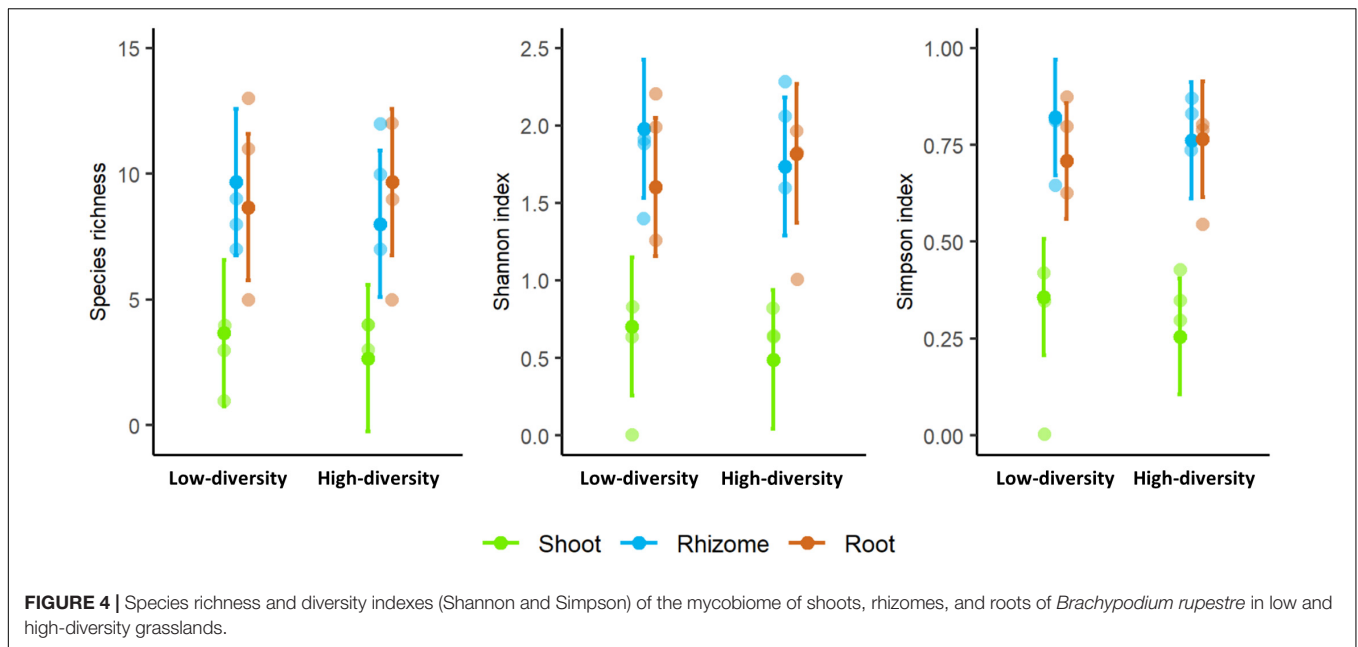


that belowground tissues have greater endophyte diversity than aerial tissues, and this could be related to the level of tissue exposition to fungal inoculum (Sánchez-Márquez et al., 2010; Harrison and Griffin, 2020). In *B. rupestre*, as in many perennial grasses, most aboveground tissues die and renew annually, while belowground tissues such as rhizomes, survive and consequently have a larger time frame for fungal reinfection.

As in other grass species, the mycobiome of *B. rupestre* was constituted by a few core species and many rare species (Comby et al., 2016; Ofek-Lalzar et al., 2016; Pereira et al., 2019; Sun et al., 2020). Only two species were found in more than 20% of the *B. rupestre* plants and in most locations, *Omnidemptus graminis* in shoots and rhizomes and *Lachnum* sp. B in roots and rhizomes. *O. graminis* is a member of the Magnaporthaceae that has been described

recently (Hernández-Restrepo et al., 2019). Although some Magnaporthaceae strains have shown plant-growth promoting activity (Yuan et al., 2010; Changyeol et al., 2017), the best known members of this family are pathogens of grasses, associated to roots (*Gaeumannomyces graminis* and *Magnaporthe poae*) and shoots (*Magnaporthe oryzae*) (Illana et al., 2013). *M. oryzae* is a hemibiotrophic fungus that causes the rice blast disease. This species switches from a biotrophic growth phase -it feeds from the host plant without killing its cells- in early infection to a necrotrophic stage (Kankanala et al., 2007). According to Talbot et al. (1997), the switch between phases may be triggered by the lack of nutrients within the host cell. In this survey, *O. graminis* was present in asymptomatic plants, suggesting at least the occurrence of a biotrophic phase of unknown duration. As many other fungal

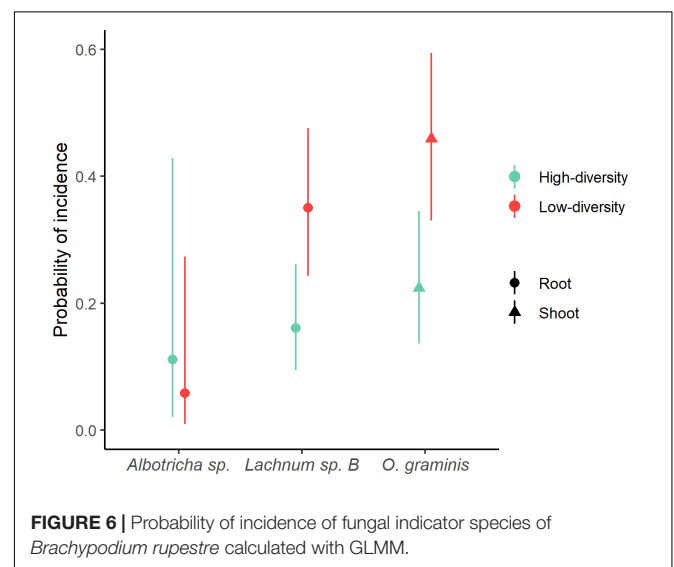
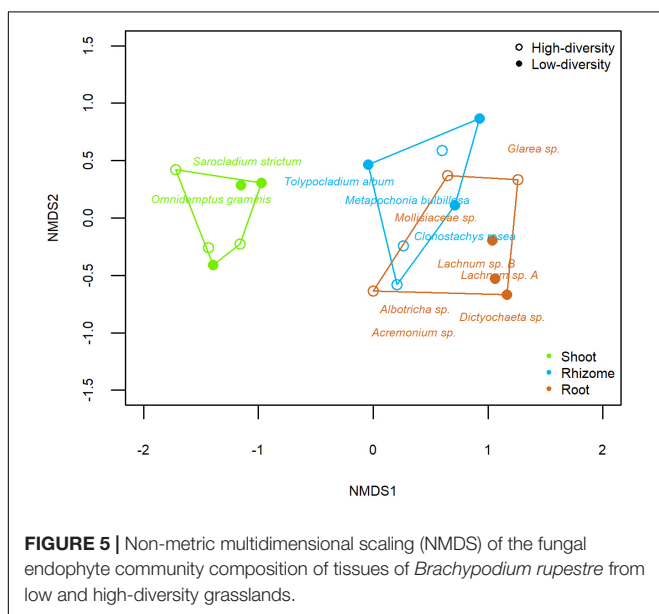




endophytes, this species may have a latent saprobic lifestyle (Vázquez-de-Aldana et al., 2013a).

*Lachnum* is a large genus within the Hyaloscyphaceae family with more than 250 species described and distributed in a wide range of habitats and host species (Wu and Su, 2007; Nagao, 2008). Most *Lachnum* species are latent saprophytes, that may grow as endophytes in roots of perennial grasses (Sánchez-Márquez et al., 2010; Pereira et al., 2019) and form ericoid mycorrhiza coils in some Ericaceae (Walker et al., 2011). Many *Lachnum* sp. are bioactive, producing a wide range of biologically active compounds (Shan et al., 1997; Ondeyka et al., 2009; McMullin et al., 2017; Zong et al., 2017).

In addition to the core species *Omnidemptus graminis* and *Lachnum* sp., the culturable mycobiota of *B. rupestre* was characterized by a remarkable number of taxa with recognized defensive activities, such as entomopathogenic fungi (*Tolypocladium album* and *Sarocladium strictum*), (Gera Hol et al., 2007; Quesada-Moraga et al., 2014; El-Sayed et al., 2020), nematophagous fungi (*Metapochonia bulbillosa* and *Clonostachys rosea*) (Sankaranarayanan et al., 1997; Ownley et al., 2010; Manzanilla-López and Lopez-Llorca, 2017), and fungi with antifungal activity (*Fusarium circinatum*, *Penicillium ortum*, *Lachnum* sp., *Glarea* sp., *Trichoderma* sp., *Trichoderma koningii* and *Nemania* sp.) (Ondeyka et al., 2009; Youssar et al., 2011; Zhang et al., 2014; Mousa et al., 2015; Kornsakulkarn et al., 2017). The known defensive functions of this group of microorganisms

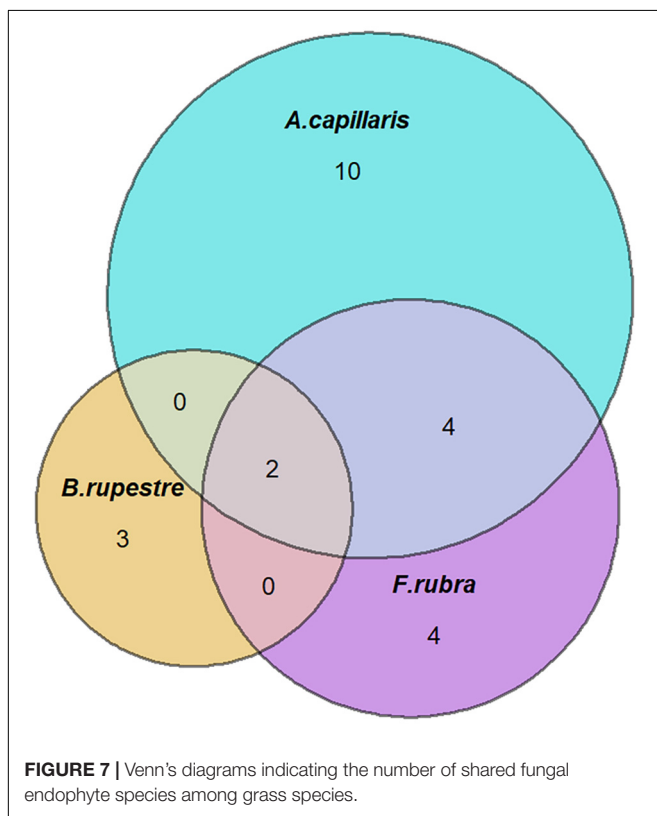


**TABLE 4** | Number of plants collected, number of final plates (taking into account the contaminations), fungal endophyte incidence (% plants), and number of fungal taxa per location for each grass species from high-diversity grasslands in aboveground tissues.

Grass species	Location	Number of Plants	Number of plates	Endophyte incidence (% plants)	Number of fungal species
<i>B. rupestre</i>	Urkulu	40	39	26	4
	Zalbetea	40	39	41	3
	Azalegi	40	38	8	1
	Total	120	116		5
<i>F. rubra</i>	Urkulu	40	39	51	7
	Zalbetea	40	38	8	1
	Azalegi	40	40	55	5
	Total	120	117		10
<i>A. capillaris</i>	Urkulu	40	40	38	7
	Zalbetea	40	40	20	8
	Azalegi	40	39	18	6
	Total	120	119		16

**TABLE 5** | Percentage of incidence of dominant fungal endophyte species in shoots of three representative grasses of high-diversity grasslands.

Grass	Fungal endophyte	Incidence (%)		
		Urkulu	Zalbetea	Azalegi
<i>B. rupestre</i>	<i>Omnidemptus graminis</i>	23.1	38.5	7.9
	<i>Epichloë typhina</i>	2.6	7.7	0
<i>F. rubra</i>	<i>Epichloë festucae</i>	23.1	0	50.0
	Mollisiaceae sp.	20.5	0	2.5
	<i>Albotricha</i> sp.	0	7.9	2.5
	<i>Alfaria dandenongensis</i>	7.7	0	0
	<i>Omnidemptus graminis</i>	0	0	5
<i>A. capillaris</i>	<i>Lachnum</i> sp. B	15.0	0	0
	<i>Epichloë baconii</i>	7.5	0	5.1
	Mollisiaceae sp.	12.5	0	0
	<i>Alfaria dandenongensis</i>	5.0	2.5	0
	<i>Ilyonectria robusta</i>	2.5	0	2.6
	<i>Albotricha</i> sp.	5.0	0	0

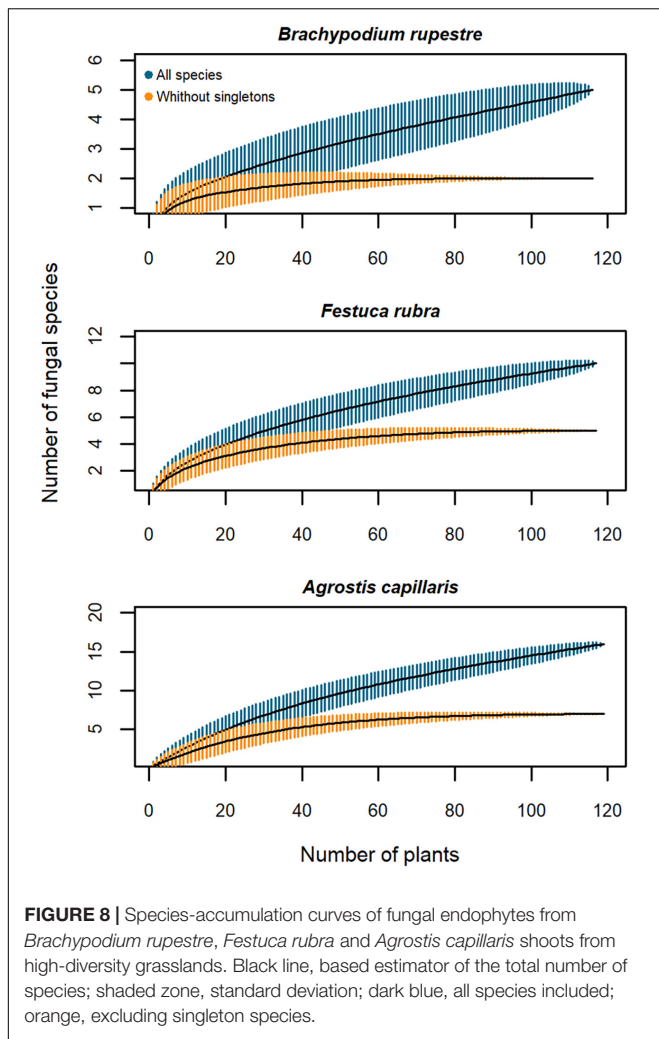


may confer a decisive advantage to *B. rupestre*, which may use them for its own protection, as predators of its pathogens and pests, according to the bodyguard hypothesis (Elliot et al., 2000). To what extent this particular defensive assemblage plays a key role in the success and expansion of *B. rupestre* with regard to other grasses is a matter of interest that needs further research.

## Fungal Assemblages in *B. rupestre* Associated to Specific Grasslands

Low and high-diversity grasslands, generated by a distinctive disturbance regime, had similar fungal richness and diversity (Figure 4), but different endophyte assemblages (Figure 2 and PERMANOVA analysis). Differences were mostly due to the different probability of incidence of the core taxa (Figure 6). *B. rupestre* in low-diversity grasslands had a greater probability of incidence of *Omnidemptus graminis* and *Lachnum* sp. B than in high-diversity grasslands, and the two grassland types only shared 36% of the total fungal taxa identified. Considering that we sampled 120 turfs per grassland type, and that we did not reach the asymptote in the species accumulation curves (Figure 3), a more exhaustive sampling effort would be necessary to draw conclusions on a specific cohort of rare taxa per grassland type. With regard to the systemic endophyte *E. typhina*, no significant differences in the probability of incidence were found between grassland types. In consequence, the highest degree of incidence of the two core taxa, *O. graminis* (in shoots and rhizomes) and *Lachnum* sp. B (in roots and rhizomes), in low-diversity, recurrently burned grasslands was the most sound result of this part of the study.

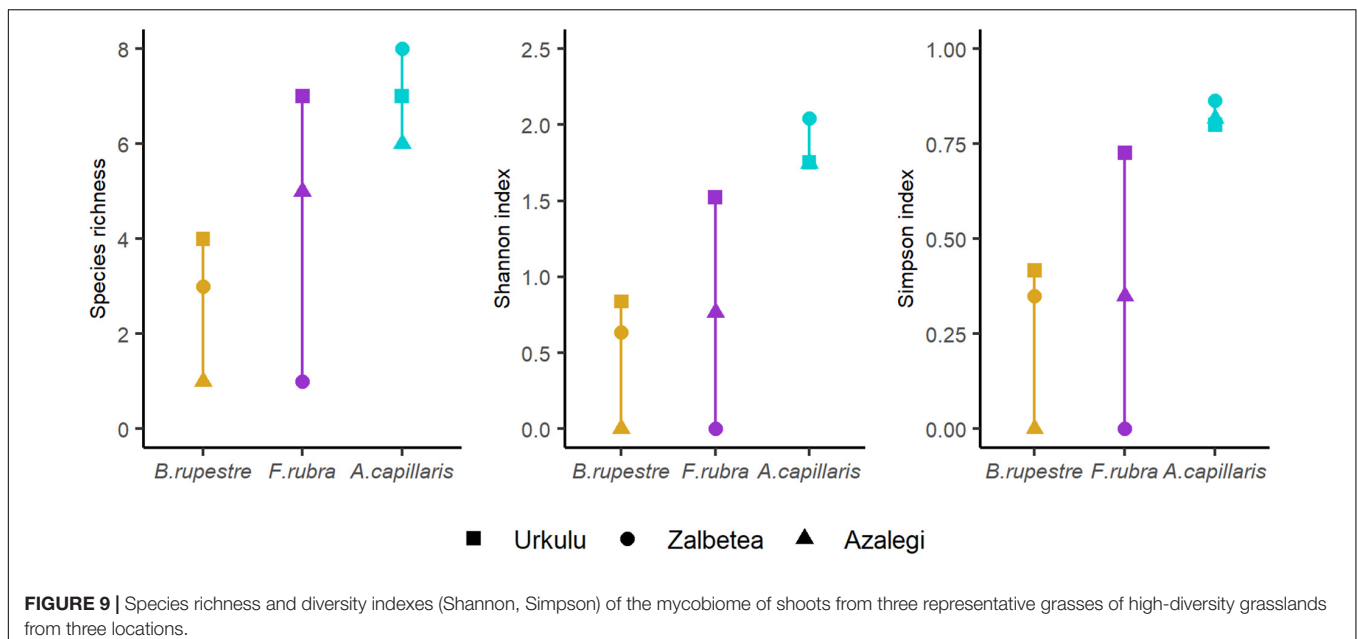
From the spectrum of core species infecting *B. rupestre*, *O. graminis* was only found in shoots and rhizomes, which indicates its affinity for both tissues, which are anatomically similar (de Kroon and Knops, 1990), and different from roots (Table 3). When isolated and cultured in plates, we observed rapid mycelial growth of *O. graminis* compared to the rest of species. We hypothesize that fires destroy the aerial mycelium of *O. graminis*, together with the aboveground biomass of the plant, but the fungus remains in the rhizome reservoir. Since fire is applied in winter time in moist soils, the temperatures reached in the top soil are low (at 1 cm deep, soil temperature rise 9–10°C and no change is measured at 5 cm depth – data unpublished), and the function of rhizomes remains unaffected.

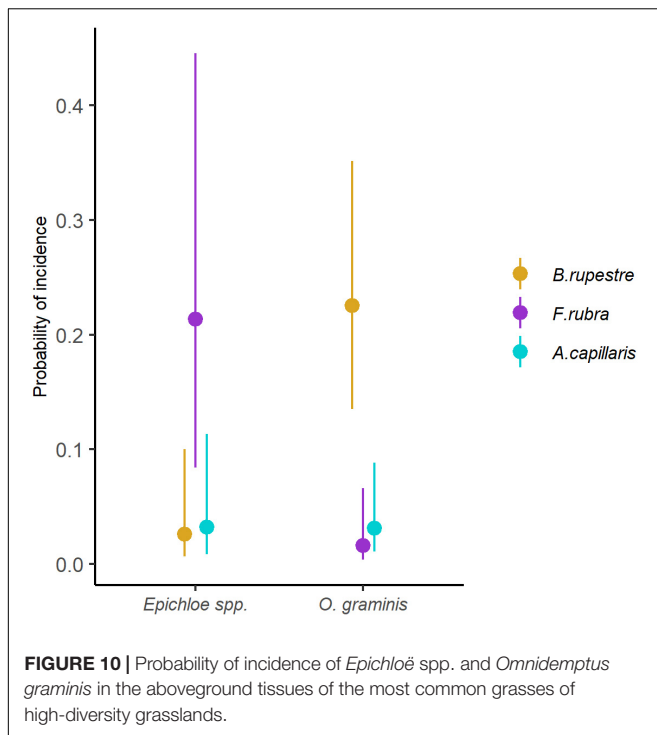


Consequently, recolonization of the aboveground tissues by the rhizome mycelium of *O. graminis* can occur rapidly, paralleling the regrowth of the plant, and conferring a decisive initial advantage to this fungal species.

Regarding *Lachnum* sp. B, the increased incidence of this fungal species in frequently burnt areas paralleled a consistent decrease of *Albotricha* sp. in roots and rhizomes (Table 3). These two endophytic taxa constitute a large part of the belowground mycobiome of *B. rupestre* and belong to the same family, Hyaloscyphaceae. The close phylogeny of both genera and the polyphyly of the *Lachnum* group has been demonstrated in genetic studies (Ye and Zhuang, 2003; Hosoya et al., 2010). The pattern of increased *Lachnum* sp. and decreased *Albotricha* sp. infection in the most burned sites, may indicate a negative interaction between both species, or a contrasting response among them to the fire disturbance. The capacity of *Lachnum* sp. to cope with environmental stress, by producing sclerotia and adopting latent forms, and its growth in belowground structures, which are less affected by fire than aboveground structures, may help to explain its success. In particular, in temperate shrublands close to the region of study, ascocarps of *Lachnum pygmaeum* have been observed on charred wood and roots of *Ulex europaeus* (unpublished results). It is known that the thermal shock produced by fire may enhance fungal fecundity and activate the development of sexual structures in some pyrophilous fungi (Raudabaugh et al., 2020). Also, fungal biomass may be increased by fires, as demonstrated for the pyrophilous fungi *Morchella* sp. infecting the grass *Bromus tectorum* (Baynes et al., 2012). To what extent the particular *Lachnum* sp. B identified in the area of study is a pyrophilous fungus merits additional study.

Although the incidences of *O. graminis* and *Lachnum* sp. B are favored in such anthropized fire-prone habitats, the extent to which these endophytes confer particular advantages to its host, *B. rupestre*, is unknown. Further experimental





research is needed to evaluate whether *O. graminis* and/or *Lachnum* sp. B infected plants perform better after fires than non-infected plants and whether the relationship in such a disrupted environment relies in a mutualistic relationship. In this research, the systemic *E. typhina* did not display significant differences in incidence among grasslands, and its percentage of infection was low at all sites. Previous research on *Epichloë* endophytes of grasses did not find evidence of a mutualistic relationship associated to fire (Faeth et al., 2002; Hall et al., 2014), contrary to grazing intensities that have been positively related to the abundance of vertically transmitted *Epichloë* producers of toxic metabolites (Vázquez-de-Aldana et al., 2010; Hume et al., 2020).

## CONCLUSION

The perennial tall-grass *B. rupestre* had a moderately diverse endophytic mycobiome consisting of a few core species and many rare species in assemblages that differed between aerial and belowground tissues. Recurrent grassland burnings, which eliminate the aerial biomass of the grass every 1–2 years, did not affect the richness and the diversity of the fungal community in *B. rupestre*, but the percentages of infection of two core taxa, *Omnidemptus graminis* and *Lachnum* sp. B, were significantly modified. The results indicate that although in frequently burnt areas the same core species of diverse grasslands subsist, *Omnidemptus graminis* and *Lachnum* sp. B are singularly benefited due to the proposed following mechanisms: (1) the capacity to survive belowground in rhizomes during the winter, and to spread rapidly to the

shoots when the plant starts its spring growth (*O. graminis*), and (2) the location in belowground tissues (*Lachnum* sp. B) and the higher resistance to stress than other core root fungi, such as the related-taxon *Albotricha* sp. Following steps should address whether these two core taxa benefit the expansive success of *B. rupestre* in these anthropized, fire-prone environments, as well as to determine whether the cohort of less abundant fungi with well-defined defensive functions play a role too.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

## AUTHOR CONTRIBUTIONS

IZ and BV introduced the training in culturable techniques for fungal identification to MD. MD and LS designed the experiments and analyzed the data. MD, LS, and LM collected the plants and isolated the fungi. MD and IZ identified the fungi. MD and RC wrote the manuscript. LS, IZ, and BV reviewed the manuscript. MD and RC got the projects that supported financially the research. All authors contributed to the article and approved the submitted version.

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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.2021.679729/full#supplementary-material>

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**Conflict of Interest:** 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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