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Spatial and temporal variation of fungal endophytic richness and diversity associated to the phyllosphere of olive cultivars

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ABSTRACT

Fungal endophytes are micro-organisms that colonize healthy plant tissues without causing disease symptoms. They are described as plant growth and disease resistance promoters and have shown antimicrobial activity. The spatial-temporal distribution of endophytic communities in olive cultivars has been poorly explored. This study aims to investigate the richness and diversity of endophytic fungi in different seasons and sites, within the Alentejo region, Portugal. Additionally, and because the impact of some pathogenic fungi (e.g. *Colletotrichum* spp.) varies according to olive cultivars; three cultivars, Galega vulgar, Cobrançosa and Azeiteira, were sampled. 1868 fungal isolates were identified as belonging to 26 OTUs; 13 OTUs were identified to the genera level and 13 to species level. Cultivar Galega vulgar and season autumn showed significant higher values in terms of endophytic richness and diversity. At site level, Elvas showed the lowest fungal richness and diversity of fungal endophytes. This study reinforces the importance of exploring the combined spatio-temporal distribution of the endophytic biodiversity in different olive cultivars. Knowledge about endophytic communities may help to better understand their functions in plants hosts, such as their ecological dynamics with pathogenic fungi, which can be explored for their use as biocontrol agents.

1. Introduction

Fungal endophytes are micro-organisms that colonize healthy plant tissues without causing disease symptoms or external structural modifications and present an ubiquitous distribution in nature (Hyde and Soytong, 2008; Kumaresan and Suryanarayanan, 2001; Pancher et al., 2012; Rodriguez et al., 2009; Schulz et al., 2002). The composition of the endophytic communities is influenced by a broad spectrum of factors such as plant physiology, around environment, pathogen infections and anthropogenic influences (Araujo et al., 2002; Buyer et al., 2011; Islam et al., 2010; Rasche et al., 2006; Saona et al., 2010; Yousaf et al., 2010). Fungal endophytes are suggested to act as plant growth promoters, to increase resistance levels to certain diseases; they also reveal antagonistic effects and antimicrobial activity through bioactive substances (Arnold et al., 2003; Bae et al., 2009; Kharwar et al., 2010; Miller et al., 2008; Oono et al., 2015; Rocha et al., 2011; Selim et al., 2011). Microorganisms that live in plant tissues, such as endophytes, have been considered as determinant factors for plant health and productivity (Berg et al., 2014), with emphasis on those living in the phyllosphere (Lindow and Brandl, 2003). The Mediterranean Basin is distinguished worldwide for the high levels of olives and olive oil production, from a wide range of olive (*Olea europaea* L.) cultivars; endophytic communities inhabiting olive tree tissues are still poorly characterized (Fisher et al., 1992; Gomes et al., 2018; Martins et al., 2016; Sia et al., 2013). In addition, studies that combine spatio-temporal variability in the endophytic community from different cultivars are practically non existent. New

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knowledge on endophytic communities is of great interest since it may help to better understand the roles of fungal endophytes in plants hosts, that include ecological dynamics with pathogenic fungi. This is particularly important because in the last decades, the use of biological agents for fungal plant pathogens control gained a considerable importance and endophytic microorganisms can be potential bio-control agents (Alabouvette et al., 2006). Recently, several studies have demonstrated that environmental conditions (humidity, rain, temperature), virulence of the pathogen, fruit maturity and integrity, combined with the type of olive cultivar, can influence abundances and diversities of the endophytic communities. These factors have also been related to the high incidence of anthracnose, the most devastating disease in the olive-producing countries (Cacciola et al., 2012; Graniti et al., 1993; Moral et al., 2008; Talhinhas et al., 2005). This disease, caused by fungi belonging to the genus *Colletotrichum* can destroy entire productions, but its impact varies according to olive cultivars. In Portugal, the main olive oil cultivar is 'Galega vulgar', which is greatly appreciated due to the unique characteristics of its olive oil but very susceptible to anthracnose. Cultivars Cobrançosa and Azeiteira are respectively considered as moderately and highly tolerant to anthracnose (Gomes et al., 2009; Talhinhas et al., 2009).

The main aim of this study was to investigate the spatial and temporal differences in patterns of endophytic fungal richness and diversity through the evaluation, under field conditions, of endophytic communities present in olive trees from three different cultivars: Galega vulgar, Cobrançosa and Azeiteira; on three different seasons: spring, summer and autumn; and grown in three distinct sites: Vidigueira, Morforte and Elvas. Consequently, it was hypothesised that this combined spatio-temporal variability could contribute to the differences in the endophytic fungi in terms of richness and diversity on the different cultivars. The following research questions were addressed: Do the endophytic fungi richness and diversity (i) vary spatially and temporally in parallel with the different sampling sites and seasons and (ii) do they vary among the different olive cultivars? Understanding the distribution patterns of the endophytic fungi and their interaction under changing conditions as proposed here, is an important baseline for ecological investigations on olive plant cultivars.

2. Materials and methods

2.1. Study area and sampling collection

Sampling was carried out during the year of 2016 in three important olive oil producing sites within Alentejo region (south of Portugal), all influenced by Mediterranean climate. The environmental parameters used in this study; temperature (°C), rainfall (mm) and relative humidity (%), were from the 30 d before the biological sampling, and are presented as mean values. In Vidigueira (38° 10′ 01.17″ N, 7° 44′ 16.75″ W) the altitude is 156 m above sea level and soils are of granite origin. The mean temperature ranged from 14.6 °C in spring to 23.6 °C in summer, the mean rainfall ranged from 0.3 mm in autumn to 3.1 mm in spring and the mean relative humidity ranged from 53.3 % in autumn to 76.2 % in spring (Table 1). In Monforte (39° 4′ 3.99″ N, 7° 28′ 13″ W) the altitude is 376 m above sea level and soils are mostly of schist and calcareous origin. The mean temperature ranged from 12.5 °C in spring to 22.6 °C in autumn, the mean rainfall ranged from 0.5 mm in autumn to 3.1 mm in spring and the mean relative humidity ranged from 56.1 % in autumn to 79.3 % in spring (Table 1). In Elvas (38° 54′ 31.34″ N, 7° 8′ 43.52″ W) the altitude is 220 m above sea level and soils are mostly of schist and calcareous origin. The mean temperature ranged from 13.8 °C in spring to 23.4 °C in autumn, the mean rainfall ranged from 0.0 mm in summer and autumn to 1.4 mm in spring and the mean relative humidity ranged from 52.2 % in autumn to 77.7 % in spring (Table 1). The ages of all olive trees sampled ranged from 10 to 30 y and trees were planted with a spacing of 7 × 5 m. Sampled olive groves occupy an area of 320.000 m^2 in Monforte, 150.000 m^2 in Vidigueira and 30.000 m^2 in Elvas and are produced under intensive regime. All experimental olive groves included programmed applications of fungicide and insecticide products such as Copper hydroxide, Trifloxystrobin, Deltamethrin and Dimethoate. Olive trees sampled belonged to three different cultivars (Galega vulgar, Cobrançosa and Azeiteira). In each site, the area of olive trees from each cultivar was divided in several plots, and three experimental plots with ten olive trees each (totaling 30 olive trees per cultivar) were randomly selected by a uniform probability function. Total fungal richness was obtained by considering the number of trees that present the fungus, out of a cluster of ten trees. A total of 270 trees were sampled (3 sites × 3 cultivars × 30 trees per cultivar). Sampling was repeated in 3 different seasons (spring, summer and autumn), totaling 810 samples (270 trees × 3 periods). Ten leaves were cut from each plant around the whole tree at 1.5 m above the ground. Sampling was always made before the applications of chemical products. Samples were transported to the laboratory in a refrigerated basket, stored at 4 °C and processed within the next 48 h.

2.2. Endophytic community – fungal isolation and DNA extraction

To suppress epiphytic micro-organisms on the field-collected samples, leaves were surface disinfected. Disinfection involved a sequence of 3 min immersions in 96 % ethanol, followed by 3 % sodium hypochlorite solution, 70 % ethanol, three times in ultrapure water and dried in sterile Whatman paper (Varanda et al., 2016). All olive leaves sampled, from each tree, were cut into small pieces of approximately 5 × 5 mm placed (six pieces per plate) on Petri dishes of 9 cm diameter containing 3.9 % of Potato Dextrose Agar medium (PDA, Merck, Germany). Flowers and drupes collected in spring and autumn were separated and discarded. The entire procedure was performed inside a sterile laminar airflow chamber. Plates were incubated in darkness at 23–25 °C for four days. The fungi that grew from the leaves sampled from each tree were then isolated by transferring a colony to a new (PDA) plate for growing. Mycelia from isolated colonies were ground in liquid nitrogen and stored at −80 °C for later use in DNA extraction for further identification of species.

The DNA extraction was done using the CTAB (Cetyltrimethyl ammonium bromide) method (Doyle and Doyle, 1987), with some modifications. Briefly, fungal powder was re-suspended in 1.5 mL microtubes with pre-warmed 600 μL of CTAB extraction buffer (20 mM EDTA, 0.1 M Tris–HCl pH 8.0, 1.4 M NaCl, 2 % CTAB, plus 4 % PVP, and 0.1 % β-mercaptoethanol added just before use) and 0.5 % Proteinase K. The solution was incubated at 55 °C for 90 min and gently mixed by inversion every 15 min. Chloroform-isoamyl alcohol (24:1) was added and the aqueous phase was transferred to a new tube following the addition of 2.5 volumes of cold ethanol (−20 °C) for nucleic acid precipitation. Samples were gently mixed and centrifuged at 10 000*g* for 20 min, washed with 500 μL of 70 % ethanol to eliminate salt residues adhered to the DNA and dried in a speed vacuum for 20 min at 50 °C.

2.3. Endophytic community – identification

Fungal isolates were identified by PCR amplification of the internal transcribed spacer (ITS) region (ITS1, 5.8S rRNA, ITS2) using ITS1 and ITS4 primers (White et al., 1990), and by amplification of part of the β-tubulin 2 (tub2) gene using T1 and T22 primers (O'Donnell and Cigelnik, 1997). PCR reactions were performed in a

Table 1
The mean values of Temperature (°C), Rainfall (mm) and Relative Humidity (%) measured at each sampling season and site.

	Spring			Summer			Autumn		
	Vidigueira	Monforte	Elvas	Vidigueira	Monforte	Elvas	Vidigueira	Monforte	Elvas
T (°C)	14.6	12.5	13.8	23.4	21.0	23.3	23.6	22.1	23.4
R (mm)	3.1	3.1	1.4	0.7	0.8	0.0	0.3	0.5	0.0
RH (%)	76.2	79.3	77.7	57.1	63.5	56.0	53.3	56.1	52.2

total volume of 50 μL, containing 30–80 ng of genomic DNA, 10 mM Tris–HCl (pH 8.6), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs (Fermentas), 0.2 μM of each primer, and 2.5 U of DreamTaq DNA polymerase (Fermentas). Amplification reactions were carried out in a Thermal Cycler (BioRad) with an initial temperature of 95 °C for 2 min followed by 40 cycles of 95 °C for 30 s, 50 °C for 50 s, and 72 °C for 60 s and a final extension at 72 °C for 10 min.

Amplified products were analyzed by agarose gel electrophoresis (1.5 % agarose gel with GelRed nucleic acid Stain) (Biotium, USA) in TBE buffer and visualized with UV light using Gel Doc (Bio Rad, USA). PCR products were purified with DNA Clean & Concentrator (Zymo Research) according to the manufacturer's instructions and sequenced in both directions by Macrogen (Spain). Sequence analysis of the ITS and tub2 sequences was carried out using MEGA 7 software (Kumar et al., 2015). The search for homologous sequences was done using Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI). Each endophytic isolate was named as taxonomic group such as a species or genus, and classified as a single Operational Taxonomic Unit (OTU). Isolates were classified into each OTU, based on a match of 100 % identity with a single species (OTU = species level) or 100 % identity to a group of similar species within the same genus (OTU = genus level).

2.4. Endophytic community – data analysis

To estimate if the number of operational taxonomical units (OTUs) obtained represented quality sampling efforts, a species accumulation curve was performed using EstimateS software (Colwell, 2013), with the protocol of randomize individuals without replacement, using the classic formula for Chao 1 and Chao 2 and Sobs (Mao Tau) algorithm. Several nonparametric estimators were used to infer species richness: Bootstrap, Chao 1 and Chao 2, Jack 1 and Jack 2, ACE and ICE estimators. Singletons and doubletons were also determined.

Univariate and multivariate analyses were performed to detect significant differences in total richness in the endophytic fungi in the olive trees on the factors season, site and cultivar. The statistical analyses of the data was performed using the PRIMER v6 software package (Clarke and Warwick, 2001) with the PERMANOVA add-on package (Anderson et al., 2008). The PERMANOVA analysis was carried out following the three factor design: Season; "Spring, Summer and Autumn" (3 levels, fixed); Site: "Vidigueira, Monforte and Elvas" (3 levels, random) and Cultivar: "Galega vulgar, Cobrançosa and Azeiteira" (3 levels, random nested in Site). A three-way permutational analysis of variance (PERMANOVA) was applied to test the hypothesis that significant differences existed in the total richness in the endophytic fungi among the factors season, cultivar and site. Total endophytic data were square root transformed in order to scale down the importance of highly abundant OTUs and therefore increase the importance of the less abundant ones in the analysis of similarity between communities. The PERMANOVA analysis was conducted on a Bray–Curtis similarity matrix (Clarke and Green, 1988). The null hypothesis was rejected at a significance level <0.05 (if the number of permutations was lower than 150, the Monte Carlo permutation p was used). Whenever significant interactions in effects of the factors were detected, these were examined using a posteriori pairwise comparisons, using 9999 permutations under a reduced model. The similarity in the endophytic fungi identified on each season, site and cultivar was plotted by Principal coordinates analysis (PCO) using the Bray–Curtis similarity measure based on each of the three factors; season, site, and cultivar. The relative contribution of each OTU to the average of similarity between a priori defined groups; season, site and cultivar; was calculated using the one way-crossed similarity percentage analysis (SIMPER, cut-off percentage: 100 %). Several indices based on diversity: Shannon–Wiener diversity (H') (Shannon and Weaver, 1963), Fisher's diversity (Fisher et al., 1943), Simpson dominance (D) (Simpson, 1949) and Pielou's evenness (J') (Pielou, 1977) were calculated using the endophytic fungi dataset from each parameter.

3. Results

3.1. Endophytic community – isolation and identification

The 270 olive trees sampled (810 field samples) harboured 1868 endophytic fungi. Fungal isolates were obtained in all samples tested. All isolated fungi were successfully identified, through the search for homologous sequences using BLAST at the NCBI, based on ITS and tub2 sequences analysis. Fungi were identified at species level in 50 % of the isolates and at genus level in 50 % of the isolates. The size of the generated PCR products ranged from 500 to 700 bp (ITS) and from 1300 to 1500 bp (tub2).

3.2. Endophytic community – structural diversity

The 1868 fungal isolates were identified as belonging to 26 OTUs; 13 OTUs were identified to the genera level and 13 identified to the species level.

The OTUs: *Alternaria* spp. showed 100 % identity with 4 species; *Alternaria alternata*, *Alternaria compacta*, *Alternaria infectoria* and *Alternaria murispora*. *Cladosporium* spp. showed 100 % identity with 5 species; *Cladosporium cladosporioides*, *Cladosporium delicatulum*, *Cladosporium herborium*, *Cladosporium pseudocladosporioides* and *Cladosporium tenellum*. *Fusarium* spp. showed 100 % identity with 4 species; *Fusarium verticillioides*, *Fusarium lateritum*, *Fusarium musae* and *Fusarium tricinctum*. *Leptosphaerulina* spp. showed 100 % identity with 4 species; *Leptosphaerulina australi*, *Leptosphaerulina americana*, *Leptosphaerulina trifolii* and *Leptosphaerulina saccharicola*. *Penicillium* spp. showed 100 % identity with 3 species; *Penicillium echinulatum*, *Penicillium expansum* and *Penicillium spinulosum*. *Peniophora* spp. showed 100 % identity with 2 species; *Peniophora cinerea* and *Peniophora lycii*. *Phoma* spp. showed 100 % identity with 2 species; *Phoma macrostoma* and *Phoma herbarum*. *Stemphylium* spp. showed 100 % identity with 2 species; *Stemphylium vesicarium* and *Stemphylium solani*.

The species accumulation curve (Fig. 1), calculated using Mao Tau algorithm, which gives confidence intervals of 95 %, indicated that the sampling efforts made were suitable to recover most of

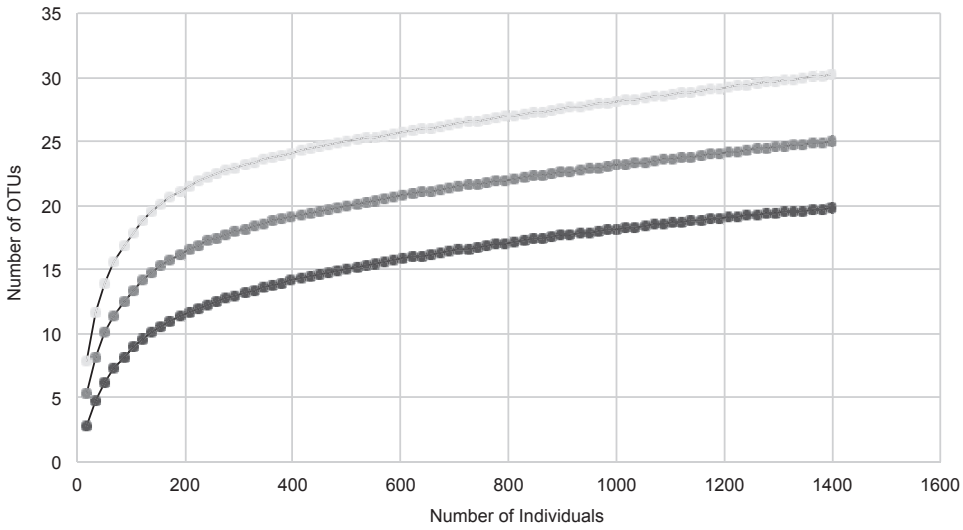

Fig. 1. Species accumulation curve showing the relation between the number of individuals (plants sampled) tested and the total number of taxa obtained. Middle line: number of fungal taxa calculated by the Mao Tau algorithm. Upper and lower lines: 95 % confidence limits of the estimate of taxa number. The curve is based on 100 randomizations.

species diversity present in the phyllospheres of the plants surveyed. The actual species number was estimated to be 27 using Bootstrap estimators, 30 using Jack 1 and ACE, 33 using ICE, 34 using Chao 2 and Jack 2 and 37 using Chao 1, meaning that the 26 OTUs found in this study represent more than 70 % of the species richness actually present. Most of the OTUs obtained in this study showed to be very frequent, 20 (77 %) appeared in four or more plants (plurals), one (4 %) in two plants (doubletons), and five (19 %) only in one plant (singletons). Overall, nearly all isolates obtained belong to Phylum Ascomycota (73.1 %), represented by four classes, with the class Dothideomycetes the most representative (34.6 %), followed by Sordariomycetes (15.4 %), Eurotiomycetes (11.5 %), Leotiomycetes (11.5 %), 11.5 % of the isolates belong to Phylum Basidiomycota, represented by two classes, class Agaricomycetes (7.7 %) and Pucciniomycetes (3.8 %). 15.4 % of the isolates obtained belong to unclassified Ascomycota. Ten genera: *Alternaria* (23.5 %), *Aureobasidium* (15.5 %), *Penicillium* (14.0 %), *Cladosporium* (12.2 %), *Biscogniauxia* (7.2 %), *Colletotrichum* (4.4 %), *Aspergillus* (4.1 %), *Botrytis* (3.7 %), *Drechslera* (2.0) and *Epicoccum* (2.9 %) together comprised nearly 90 % of the total fungal diversity.

At the season level, the number of OTUs identified in spring was 11 and 100 % belonged to the phylum Ascomycota represented by three classes; with the class Dothideomycetes being the most representative (54.5 %), followed by Sordariomycetes (18.2 %) and Eurotiomycetes (9.1 %). 18.2 % of the isolates obtained belong to unclassified Ascomycota. SIMPER analysis revealed that, from the 11 OTUs identified in Spring, six OTUs; *Alternaria* spp. (51.3 %), *Aureobasidium pullulans* (16.4 %), *Biscognauxia mediterranea* (8.5 %), *Cladosporium* spp. (7.9 %), *Colletotrichum nymphaeae* (2.8 %) and *Drechslera avenae* (1.0 %) represent 87.9 % of the similarities (Table 2).

The number of OTUs identified in summer was 18, from which 77.8 % belonged to the phylum Ascomycota and were represented by four classes; with the class Dothideomycetes being the most representative (44.4 %), followed by Sordariomycetes (22.2 %), Eurotiomycetes (5.6 %) and Leotiomycetes (5.6 %). Two OTUs representing 11.1 % of the isolates, belong to class Agaricomycetes (5.6 %) and Pucciniomycetes (5.6 %) both belonging to the phylum Basidiomycota. 11.2 % of the isolates obtained belong to unclassified Ascomycota. SIMPER analysis revealed that, from the 18 OTUs identified in summer, six OTUs; *Alternaria* spp. (37.3 %), *A. pullulans* (18.5 %), *B. mediterranea* (17.2 %), *Cladosporium* spp. (12.8 %), *Penicillium* spp. (7.1 %) and *C. nymphaeae* (3.3 %), represented 96.1 % of the similarities (Table 2).

The number of OTUs identified in autumn was 22, from which 73.3 % belonged to the phylum Ascomycota and were represented by four classes; with the class Dothideomycetes being the most representative (34.6 %), followed by Eurotiomycetes (13.6 %), Sordariomycetes (13.6 %) and Leotiomycetes (13.6 %). One OTU representing 4.5 % of the isolates, belong to class Agaricomycetes, phylum Basidiomycota. 18.2 % of the isolates obtained belong to unclassified Ascomycota. SIMPER analysis revealed that, from the 22 OTUs identified in Autumn, six OTUs; *Alternaria* spp. (15.7 %), *Cladosporium* spp. (18.4 %), *A. pullulans* (13.2 %), *Penicillium* spp. (12.8 %), *Botrytis cinerea* (6.3 %), *Aspergillus* (5.7 %) represent 72.0 % of the similarities (Table 2).

At the cultivar level, the number of OTUs identified in Galega vulgar was 22, from which 77.3 % belonged to the phylum Ascomycota and were represented by four classes; with the class Dothideomycetes being the most representative (40.9 %), followed by Sordariomycetes (13.6 %), Leotiomycetes (13.6 %) and Eurotiomycetes (9.1 %). One OTU representing 4.5 % of the isolates, belong to class Agaricomycetes, phylum Basidiomycota. 18.1 % of the isolates obtained belong to unclassified Ascomycota. SIMPER analysis showed that, from the 22 OTUs identified in Galega vulgar, six OTUs; *Alternaria* spp. (41.3 %), *A. pullulans* (15.9 %), *B. mediterranea* (9.9 %), *Penicillium* spp. (9.3 %), *C. nymphaeae* (8.9 %) and *Cladosporium* spp. (8.5 %), represented 93.8 % of the similarities (Table 2).

The number of OTUs identified in Cobrançosa was 20, from which 85.0 % belonged to the phylum Ascomycota and were represented by four classes; with the class Dothideomycetes being the most representative (40.0 %), followed by Sordariomycetes (20.0 %), Eurotiomycetes (15.0 %) and Leotiomycetes (10.0 %). 15.0 % of the isolates obtained belong to unclassified Ascomycota. SIMPER analysis showed that, from the 20 OTUs identified in Cobrançosa, six OTUs; *Alternaria* spp. (34.7 %), *Cladosporium* spp. (24.9 %), *A. pullulans* (23.9 %), *B. mediterranea* (6.6 %), *Penicillium* spp. (5.1 %), *Epicoccum nigrum* (0.9 %) represented 96.0 % of the similarities (Table 2).

The number of OTUs identified in Azeiteira was 20, from which 80.0 % belonged to the phylum Ascomycota and were represented by four classes; with the class Dothideomycetes being the most representative (40.0 %), followed by Sordariomycetes (15.0 %), Eurotiomycetes (10.0 %) and Leotiomycetes (10.0 %). One OTU

Table 2
Fungal OTUs that contribute most to the similarities identified by SIMPER analysis. A) Distinguishing the OTUs present in Seasons (Spring, Summer and Autumn); B) Distinguishing the OTUs present in Cultivar (Galega, Cobrançosa and Azeiteira); and C) Distinguishing the OTUs present in Site (Vidigueira, Morforte and Elvas).

OTUs	Season			Site			Cultivar		
	Spring	Summer	Autumn	Vidigueira	Monforte	Elvas	Galega vulgar	Cobrançosa	Azeitera
	%			%			%		
Number of OTUs	**11**	**18**	**22**	**22**	**21**	**16**	**22**	**20**	**20**
Alternaria spp.	51.3	37.3	15.7	30.7	42.3	31.8	41.3	34.7	30.1
Aureobasidium pullulans	16.4	18.5	13.2	30.9	16.1	6.9	15.9	23.9	12.4
Biscognauxia mediterranea	8.5	17.2	3.4	4.2	5.5	22.7	9.9	6.6	13.2
Penicillium spp.	9.8	7.1	12.8	11.2	11.1	8.6	9.3	5.1	19.6
Colletotrichum nymphaeae	2.8	3.3	5.3	1.6	2.2	10.1	8.9	0.3	6.8
Cladosporium spp.	7.9	12.8	18.4	12.0	11.6	15.2	8.5	24.9	8.9
Epicoccum nigrum	–	0.3	5.2	3.3	0.6	–	1.6	0.9	0.5
Botrytis cinerea	–	0.0	6.3	2.0	1.0	–	1.4	0.6	0.4
Drechslera avenae	1.0	2.8	–	0.4	5.8	–	0.9	0.7	1.2
Fusarium spp.	–	–	1.0	0.2	0.3	–	0.6	0.1	–
Gloeotinia temulenta	–	–	4.3	0.5	0.3	0.9	0.6	0.3	0.9
Rhizopus spp.	–	–	4.8	0.9	1.1	–	0.5	0.6	0.6
Aspergillus spp.	–	–	5.7	–	0.4	2.9	0.3	0.5	1.3
Pteris vitata	2.4	0.2	–	1.7	0.2	0.1	0.2	0.2	1.7
Peniophora spp.	–	–	–	–	–	–	–	–	–
Claetomium aureum	–	–	–	–	–	–	–	–	–
Preussia africana	–	0.4	–	0.3	–	–	–	0.1	0.1
Leptosphaerulina spp.	–	0.1	–	–	0.2	–	–	0.1	0.1
Phoma spp.	–	–	3.5	0.1	1.3	0.7	–	0.5	2.3
Phyctema Vagabunda	–	–	–	–	–	–	–	–	–
Stemphylium spp.	–	0.1	0.3	0.1	0.2	–	–	0.1	0.1
Neofabraea	–	–	–	–	–	–	–	–	–
Neosartorya	–	–	–	–	–	–	–	–	–
Pelosfora spp.	–	–	–	–	–	–	–	–	–
Phlebiopsis gigantea	–	–	–	–	–	–	–	–	–
Rhodotorula mucilaginosa	–	–	–	–	–	–	–	–	–

Bold values highlight the number of OTUs obtained in each season, site and cultivar.

representing 5.0 % of the isolates, belong to class Pucciniomycetes, phylum Basidiomycota. 15.0 % of the isolates obtained belong to unclassified Ascomycota. SIMPER analysis showed that, from the 20 OTUs identified in Azeiteira, six OTUs; *Alternaria* spp. (30.1 %), *Penicillium* spp. (19.6 %), *B. mediterranea* (13.2 %), *A. pullulans* (12.4 %), *Cladosporium* spp. (8.9 %) *and C. nymphaeae* (6.8 %), represented 91.0 % of the similarities (Table 2).

At the site level, the number of OTUs identified in Vidigueira was 22, from which 77.3 % belonged to the phylum Ascomycota and were represented by four classes; with the class Dothideomycetes being the most representative (40.9 %), followed by Sordariomycetes (18.2 %), Eurotiomycetes (9.1 %) and Leotiomycetes (9.1 %). Two OTUs representing 9.1 % of the isolates belong to class Agaricomycetes (4.5 %) and Pucciniomycetes (4.5 %), phylum Basidiomycota. 13.6 % of the isolates obtained belong to unclassified Ascomycota. SIMPER analysis showed that, from the 22 OTUs identified in Vidigueira, six OTUs; *A. pullulans* (30.9 %), *Alternaria* spp. (30.7 %), *Cladosporium* spp. (12.0 %), *Penicillium* spp. (11.2 %), *B. mediterranea* (4.2 %), *E. nigrum* (3.3 %), showed 92.3 % of the similarities (Table 2).

The number of OTUs identified in Monforte was 21, from which 81.0 % belonged to the phylum Ascomycota and were represented by four classes; with the class Dothideomycetes being the most representative (42.9 %), followed by Sordariomycetes (14.3 %), Leotiomycetes (14.3 %) and Eurotiomycetes (9.5 %). However, 19.0 % of the isolates obtained belong to unclassified Ascomycota. SIMPER analysis showed that, from the 21 OTUs identified in Monforte, six OTUs; *Alternaria* spp. (42.3 %), *A. pullulans* (16.1 %), *Cladosporium* spp. (11.6 %), *Penicillium* spp. (11.1 %), *D. avenae* (5.8 %), *B. mediterranea* (5.5 %), represented 92.4 % of the similarities (Table 2).

The number of OTUs identified in Elvas was 16, from which 81.3 % belonged to the phylum Ascomycota and were represented by four classes; with the class Dothideomycetes being the most representative (31.3 %), followed by Eurotiomycetes (18.8 %), Sordariomycetes (18.8 %) and Leotiomycetes (12.5 %). One OTU representing 6.3 % of the isolates, belong to class Agaricomycetes (4.5 %), phylum Basidiomycota. 12.5 % of the isolates obtained belong to unclassified Ascomycota. SIMPER analysis showed that, from the 16 OTUs identified in Elvas, six OTUs; *Alternaria* spp. (31.8 %), *B. mediterranea* (22.7 %), *Cladosporium* spp. (15.2 %), *C. nymphaeae* (10.1 %), *Penicillium* spp. (8.6 %), *A. pullulans* (6.9 %), represented 95.2 % of the similarities (Table 2).

Diversity based on Shannon–Wiener values (*H'*) (Fig. 2) showed significant differences for factor season ($p = 0.0463$) and "site" ($p = 0.022$) (Table 3). Despite not differing substantially, diversity among olive trees across different seasons was higher in autumn when compared to spring (Pairwise Tests, $p_{\text{Autumn vs. Spring}} = 0.0383$). The diversity differed considerably among sites, diversity was significant higher in Vidigueira and Monforte than in Elvas (Pairwise Tests, $p_{\text{Vidigueira vs. Elvas}} = 0.005$; $p_{\text{Monforte vs. Elvas}} = 0.0102$), and between Vidigueira and Monforte no significant differences existed (Pairwise Tests, $p_{\text{Vidigueira vs. Monforte}} = 0.5896$).

Season ($p = 0.0121$) and site ($p = 0.0241$) affected significantly fungal diversity based on Fisher's alpha Index (Fig. 2) (Table 3). Diversity was higher in autumn in comparison to spring and summer and Vidigueira was significantly higher in comparison to Monforte and Elvas (Pairwise Tests, $p_{\text{Vidigueira vs. Monforte}} = 0.0482$ and $_{\text{Vidigueira vs. Elvas}} = 0.0153$).

Season and cultivar affected significantly ($p = 0.0103$ and $p = 0.0113$, respectively) fungal dominance based on Simpson index (Fig. 3) (Table 3). While diversity did not differ substantially among olive trees across seasons, diversity was higher in autumn and summer in comparison to spring (Pairwise Tests, $p_{\text{Autumn vs. Spring}} = 0.0337$; $p_{\text{Summer vs. Spring}} = 0.024$). Fungal endophytic diversity was significant higher in Galega vulgar when compared to Cobrançosa in Vidigueira (Pairwise Tests, $p_{\text{Galega vs.}}$

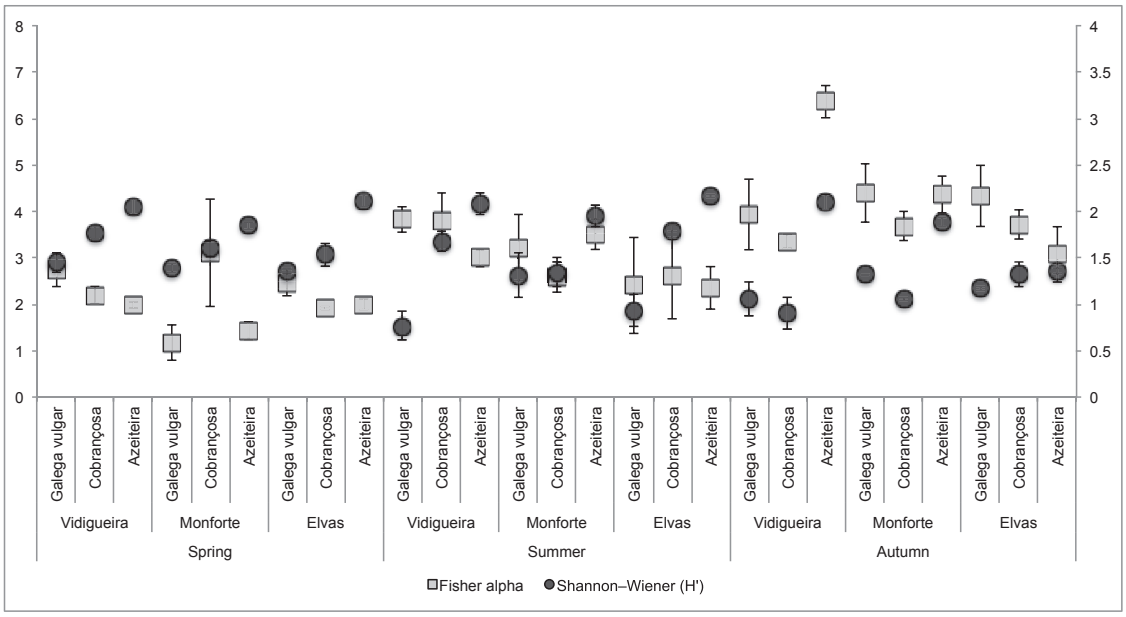

Fig. 2. Mean fungal ± standard error (SE) of Shannon–Wiener index (*H'*) and Fisher's alpha indices at each season (Spring, summer and autumn), site (Vidigueira, monforte and elvas) and cultivar (Galega vulgar, cobrançosa and azeiteira).

Table 3
Details of the three-factor PERMANOVA test on the endophytic fungal dataset for the factors; "Season" Spring, Summer and Autumn (3 levels, fixed), "Site" Vidigueira, Monforte and Elvas (3 levels, random), "Cultivar" Galega, Cobrançosa and Azeiteira (3 levels, random nested in "Site") for all variables analyzed.

	Source of variation	Degrees of freedom	Sum of squares	Mean squares	Pseudo-F	perms	P (perm)
Fungal richness	Site	2	18 566	9282.8	3.9459	280	**0.0054**
	Season	2	29 978	14 989	3.6882	6115	**0.0123**
	Cultivar (Site)	6	14 115	2352.5	4.4701	9906	**0.0001**
	Season × Site	4	16 256	4064.1	4.4855	9924	**0.0001**
	Cutivar (Site) × Season	12	10 873	906.05	1.7216	9880	**0.0069**
	Residual	54	28 420	526.29			
	Total	80	118 210				
Shannon–Wiener index	Site	2	407.14	203.57	11.128	280	**0.0059**
	Season	2	2451.3	1225.6	7.5498	6141	**0.0327**
	Cultivar (Site)	6	109.76	18.293	0.96764	9937	0.4699
	Season × Site	4	649.36	162.34	3.6153	9964	**0.0325**
	Cutivar (Site) × Season	12	538.84	44.903	2.3753	9928	**0.0131**
	Residual	54	1020.8	18.904			
	Total	80	5177.2				
Fisher index	Site	2	511.14	255.57	6.6372	280	**0.0241**
	Season	2	3981.6	1990.8	12.743	6150	**0.0121**
	Cultivar (Site)	6	231.03	38.505	0.60922	9946	0.744
	Season × Site	4	624.91	156.23	1.3443	9955	0.2995
	Cutivar (Site) × Season	12	1394.6	116.21	1.8387	9916	0.06
	Residual	54	3413	63.204			
	Total	80	10 156				
Simpson index	Site	2	78.962	39.481	7.9706	280	**0.0177**
	Season	2	486.31	243.16	3.6376	6106	0.1255
	Cultivar (Site)	6	29.72	4.9533	0.50641	9949	0.8133
	Season × Site	4	267.38	66.845	5.532	9958	**0.0094**
	Cutivar (Site) × Season	12	145	12.083	1.2353	9939	0.2862
	Residual	54	528.19	9.7813			
	Total	80	1535.6				
Pielou's evenness index	Site	2	11.177	5.5885	0.71908	280	0.5306
	Season	2	34.085	17.042	1.1978	6117	0.3933
	Cultivar (Site)	6	46.631	7.7718	2.5543	9953	**0.0292**
	Season × Site	4	56.91	14.228	2.1378	9965	0.14
	Cutivar (Site) × Season	12	79.862	6.6551	2.1873	9946	**0.025**
	Residual	54	164.3	3.0426			
	Total	80	392.97				

Bold values highlight significant effects and interactions ($p < 0.05$).

$_{Cobrançosa} = 0.0068$), and to Azeiteira in Elvas (Pairwise Tests, $p_{Galega\ vs.\ Azeiteira} = 0.0229$).

The fungal evenness estimated by Pielou's (J') index was only significantly ($p = 0.0001$) affected by the factor "cultivar" (Fig. 3) (Table 3). Although the variability of the cultivars was high between sites, when cultivars were compared within each site, diversity did not differ substantially, and a significant higher evenness was only observed in Galega vulgar when compared to Azeiteira (Pairwise

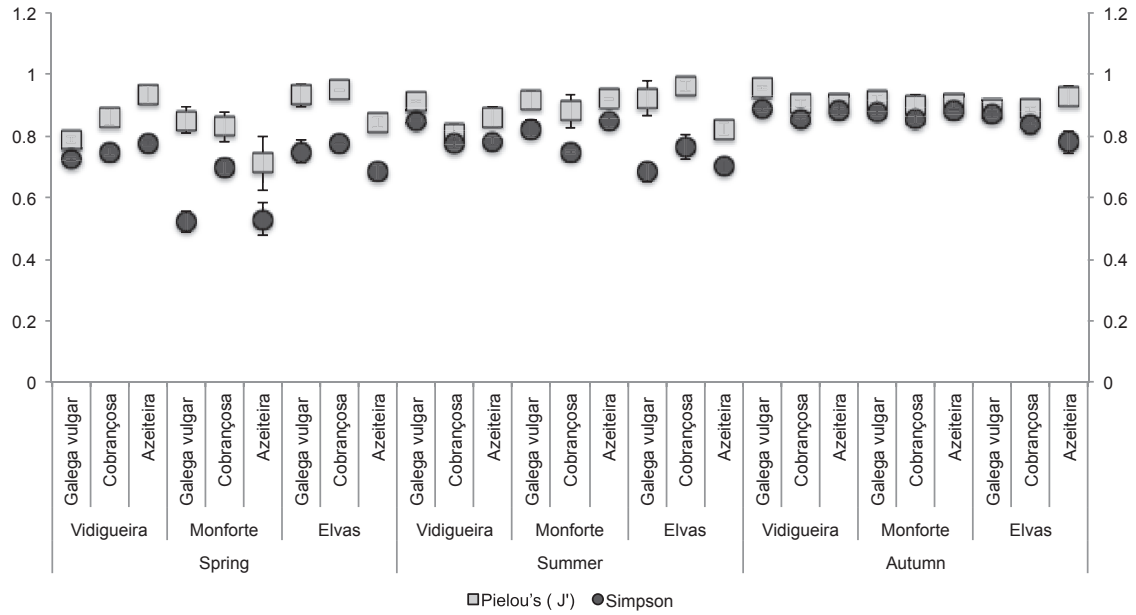

Fig. 3. Mean fungal ± standard error (SE) of Pielou's (*J'*) and Simpson indices at each season (Spring, summer and autumn), site (Vidigueira, monforte and elvas) and cultivar (Galega vulgar, cobrançosa and azeiteira).

Tests, p $_{\text{Galega vs. Azeiteira}}$ = 0.0002) and in Cobrançosa when compared to Azeiteira (Pairwise Tests, p $_{\text{Cobrançosa vs. Azeiteira}}$ = 0.0005), both in Elvas.

3.3. Endophytic community – multivariate data analysis

The mean fungal richness ± SE (per 10 trees) was 15.5 ± 1.1 in spring, 17.0 ± 1.4 in summer and 36.7 ± 2.7 in autumn (Fig. 4). PERMANOVA analyses showed significantly higher fungal richness in autumn (factor "Season", p = 0.0075) (Table 3) when compared to spring and summer. No significant differences (p = 0.5339) in fungal richness were detected between spring and summer. These results are also supported by the PCO ordination plot and clearly reflect a distinct pattern for endophytic richness in autumn compared to spring and summer. The PCO ordination of the endophytic richness showed that the first two components (PCO1, 28.2 % and PCO2, 22.7 %) accounted for 50.9 % of the variability of the data (Fig. 5).

The mean fungal richness ± SE (per 10 trees) at site level was 23.8 ± 1.3 in Vidigueira, 25.8 ± 3.4 in Monforte and 19.7 ± 2.6 in Elvas (Fig. 4). PERMANOVA showed significant differences in fungal richness on factor "site" (p = 0.0043) (Table 3). Individual pairwise comparisons confirmed the high variability in terms of fungal endophytic richness in Elvas when compared to Vidigueira and Monforte (Pairwise Tests, p $_{\text{Vidigueira vs. Elvas}}$ = 0.0088). (Pairwise Tests, p $_{\text{Monforte vs. Elvas}}$ = 0.0364). These results are supported by the PCO analysis that confirms a high variability between-sites of endophytic richness and clearly reflect a distinct pattern of "Elvas" from "Vidigueira" and "Monforte". The PCO ordination of the endophytic richness showed that the first two components (PCO1, 28.2 % and PCO2, 22.7 %) accounted for 50.9 % of the variability of the data (Fig. 6).

The mean fungal richness ± SE (per 10 trees) at cultivar level was 23.2 ± 2.6 in Galega vulgar, 21.1 ± 1.9 in Cobrançosa and 24.9 ± 3.2 in Azeiteira (Fig. 4). The variation of the endophytic richness between cultivars and sites ("cultivar" nested in "site") showed significant differences (p = 0.0001) (Table 3). In Vidigueira the mean fungal richness ±SE was 26.3 ± 2.5 in Galega followed by 24.3 ± 2.0 in Cobrançosa and 20.7 ± 2.1 in Azeiteira (Fig. 4). Individual pairwise comparisons for endophytic richness revealed high variability between cultivars (factor "cultivar" nested in "site") at Vidigueira, with significant higher richness in Galega when compared to Cobrançosa (Pairwise Tests, p $_{\text{Galega vs. Vidigueira}}$ = 0.0004) as well as in Galega when compared to Azeiteira (Pairwise Tests, p $_{\text{Galega vs. Azeiteira}}$ = 0.0029). No significant differences were observed between cultivars Cobrançosa and Azeiteira (Pairwise Tests, p $_{\text{Galega vs. Azeiteira}}$ = 0.0658). In Monforte the mean endophytic richness ±SE was 22.75 ± 3.8 in Galega vulgar followed by 21.4 ± 3.7 in Cobrançosa and 33.1 ± 8.8 in Azeiteira (Fig. 4). Individual pairwise comparisons at Monforte showed significant higher endophytic richness in Galega vulgar when compared to Cobrançosa (Pairwise Tests, p $_{\text{Galega vs. Vidigueira}}$ = 0.0012), in Azeiteira when compared to Galega (Pairwise Tests, p $_{\text{Azeiteira vs. Galega}}$ = 0.0064) and in Azeiteira when compared to Cobrançosa (Pairwise Tests, p $_{\text{Azeiteira vs. Cobrançosa}}$ = 0.0004). In Elvas the mean fungal richness ±SE was 20.5 ± 6.7 in Galega vulgar followed by 17.5 ± 3.7 in Cobrançosa and 21.0 ± 2.8 in Azeiteira (Fig. 4). Individual pairwise comparisons also showed significant higher endophytic richness in Galega vulgar than in Cobrançosa (Pairwise Tests, p $_{\text{Galega vs. Vidigueira}}$ = 0.0037), as well as in Galega vulgar when compared to Azeiteira (Pairwise Tests, p $_{\text{Galega vs. Azeiteira}}$ = 0.0055) and in Cobrançosa when compared to Azeiteira (Pairwise Tests, p $_{\text{Galega vs. Azeiteira}}$ = 0.0002). These results are also supported by PCO ordination plot and clearly reflect the high variability on factor "cultivar". The PCO ordination of the endophytic richness showed that the first two components (PCO1, 28.2 % and PCO2, 22.7 %) accounted for 50.9 % of the variability of the data (Fig. 7).

4. Discussion

This study describes the composition of endophytic fungal communities within the phyllosphere of olive trees from different cultivars, in different seasons and sites located in the Alentejo region, the main olive producing region, located in the South of Portugal. Until now, no studies have been conducted in olive (*O. europaea* L.) combining spatio-temporal variability of the richness and diversity of endophytic fungi. Overall, in the present study, 26

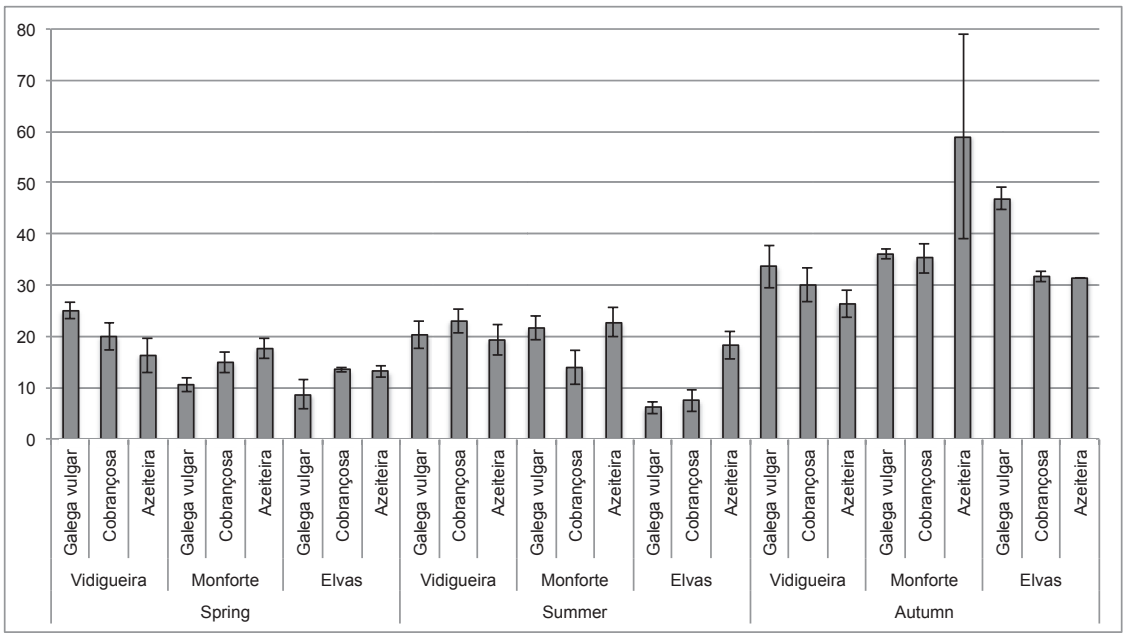

Fig. 4. Mean endophytic fungal richness ± standard error (SE) at each season (Spring, summer and autumn), site (Vidigueira, monforte and elvas) and cultivar (Galega vulgar, cobrançosa and azeiteira).

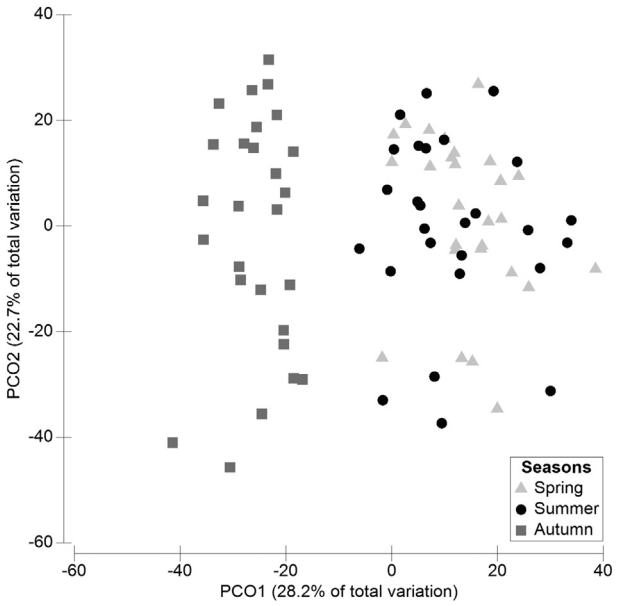

Fig. 5. Principal coordinates analysis PCO based on the endophytic fungal richness dataset for the factors "Seasons" Spring, Summer and Autumn (3 levels, fixed). PCO1 = 23.8 % and PCO2 = 20.6 %.

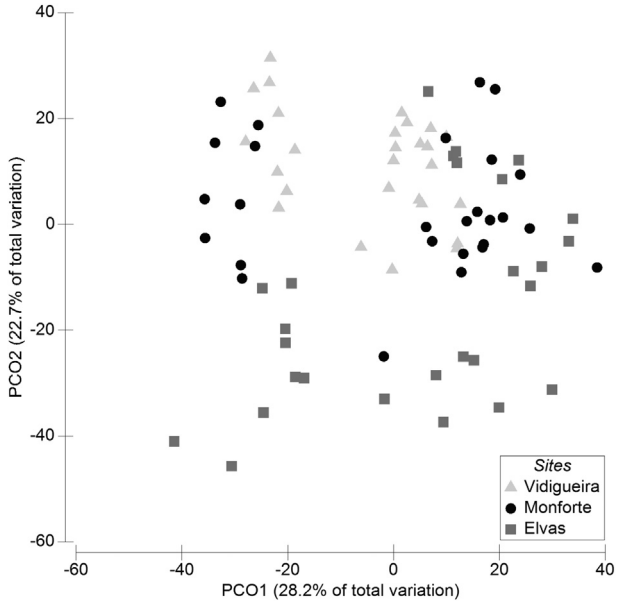

Fig. 6. Principal coordinates analysis PCO based on the endophytic fungal richness dataset for the factors "Cultivar" Galega vulgar, Cobrançosa and Azeiteira (3 levels, random nested in "Site"). PCO1 = 31.7 % and PCO2 = 18.6 %.

endophytic OTUs were characterized; 13 were identified to the genera level and 13 to the species level, from a total of 270 trees, representative of Alentejo. The present results reveal higher values in terms of endophytic fungal diversity than the ones obtained by Fisher et al. (1992), but lower than the ones obtained by Martins et al. (2016) and Gomes et al. (2018), all in *O. europaea* L. These differences in the endophytic communities may be associated with several factors such as: an underestimated fungal diversity due to low spatial–temporal sampling, the type of vegetative tissue (e.g. leaves, twigs, flowers, branches, fruits), endophytic or epiphytic communities of these tissues and environmental factors at sample sites.

Ascomycota represents the majority (73.1 %) of the identified endophytic taxa in this study, being the dominant Phylum found in all cultivars, seasons and sites. This seems to be a general characteristic of the endophytic communities in olive as well as in other plants (Fisher et al., 1992; Martins et al., 2016; Moricca et al., 2012; Varanda et al., 2016). The low proportions of Basidiomycota (11.5 %) could probably reflect sampling bias (Mueller et al., 2004; Pinruan et al., 2010), but studies in olive trees with an acceptable robustness

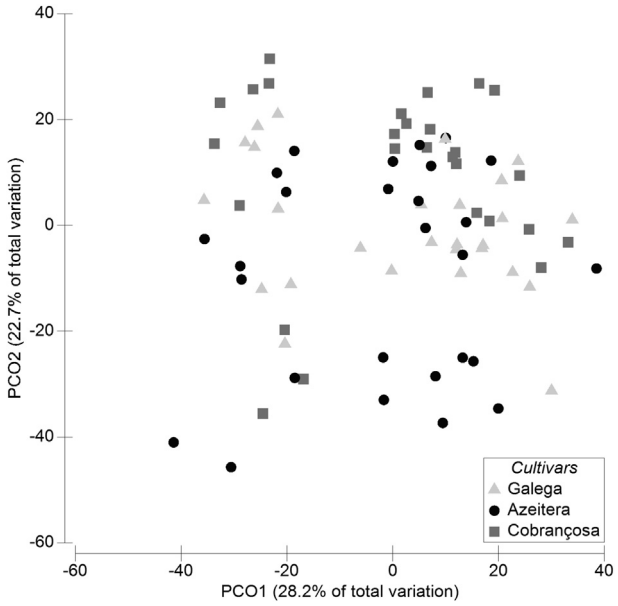

Fig. 7. Principal coordinates analysis PCO based on the endophytic fungal richness dataset for the factors "Site" Vidigueira, Monforte and Elvas (3 levels, random). PCO1 = 23.8 % and PCO2 = 20.6 %.

of the sampling confirmed similar results (Gomes et al., 2018; Martins et al., 2016).

In general, the genera *Alternaria* (23.5 %), *Aureobasidium* (15.5 %), *Penicillium* (14.0 %), *Cladosporium* (12.2 %), *Biscognauxia* (7.2 %), *Aspergillus* (4.1 %), *Colletotrichum* (4.4 %), *Botrytis* (3.7 %), *Epicoccum* (2.9 %), *Rhizopus* (2.5 %), *Drechslera* (2.0 %), *Phoma* (1.8 %), *Gloeotinia* (1.8 %) and *Pteris* (1.3 %) together comprised 97.0 % of the total fungal diversity, most of them already referred as leading the diversity in olive tree (Abdelfattah et al., 2015; Fisher et al., 1992; Gomes et al., 2018; Martins et al., 2016; Sia et al., 2013). *Alternaria* spp. was the OTU that most contributed to the similarities in season, site and cultivar. The aforementioned OTU is commonly the principal component of endophytic communities in olive phyllospheres, likely due to their particular life style, which includes producing highly melanised hyphae capable to resist and grow under intense UV radiations (Fisher et al., 1992; Gomes et al., 2018; Martins et al., 2016; Sia et al., 2013). Recently, a frequent asymptomatic olive endophytic fungus *A. alternata*, has been described as pathogenic and responsible for high losses in Turkey (Basim et al., 2017) and Greece (Lagogianni, 2017), whilst no symptoms of pathogenicity caused by the *A. alternata* have been seen in the olive trees sampled for this study. *B. mediterranea* showed high presence in the present study, this fungus is also described as a relevant plant pathogen but did not cause any visible symptoms of pathogenicity. In fact, many fungal pathogens present endophytism as a common phase in the life cycle (Carroll, 1988; Joshee et al., 2009; Rodriguez and Redman, 1997; Schulz and Boyle, 2005) and asymptomatic endophytes can easily switch to necrotrophism (Delaye et al., 2013). *Cladosporium* spp. together with *A. pullulans* are described as secondary invaders of necrotic tissues or as weak pathogens in many different host plants and occur as cosmopolitan saprophytic fungi (Tashiro et al., 2013). In favorable conditions, both fungal species may produce a compact sooty thallus on the fruit surface, which can change the olive fruit quality (Grabowski, 2007). Nevertheless both *A. pullulans* and *C. cladosporioides* have been reported as effective bio-control agents (Wang et al., 2013).

The spatial and temporal distribution of endophytic communities revealed significant differences in terms of endophytic richness and diversity according to cultivar, season and site. In terms of season, the diversity of fungal communities was shaped by autumn, showing consistently significant higher fungal diversity and dominance. Fungal endophytic richness also showed higher values in autumn. Indeed, the changes on the environmental factors across seasons have been described as the major drivers that shape endophytic fungal communities (Fisher et al., 1992; Gomes et al., 2018; Martins et al., 2016; Sia et al., 2013). Despite the differences in the sampling robustness between studies, the highest diversity and fungal richness in autumn is surprising compared to other works, which seem to find highest diversity and fungal richness in spring (Collado et al., 1999; Martins et al., 2016; Gomes et al., 2018). Some authors suggest that both rainfall and humidity are the key factors for the pattern of endophytes, shaping communities due to their importance on fungal spores dispersion and colonization (Rastogi et al., 2012; Gomes et al., 2018; Martínez-Álvarez et al., 2012; Vacher et al., 2016). The sampling sites are usually rainier in autumn and the humidity created by the rain in this season (Gomes et al., 2018) may be the explanation for the highest endophytic diversities and richness in this season. In addition, the presence of endophytes and other fungi in leaf litter, may be particularly relevant to seasonal differences in endophyte communities (Christian et al., 2017). Despite olive fruits were not used for this study, their presence since the early stages on trees can be one of the factors indirectly shaping the high species richness in autumn, due to the increased nutrient conditions associated with the high humidity, which can benefit the endophytic community in other plant tissues (e.g. leaves) (Rastogi et al., 2012). As previously stated, rain and high humidity have a direct effect on both endophytic fungal colonization and dispersion (Gomes et al., 2018), which may also help to explain why Elvas showed the lowest fungal endophytic diversities and richness when compared to Vidigueira and Monforte, as Elvas presents the lowest values of rainfall and relative humidity. Conclusions made by Bokulich et al. (2014) reveal that nonrandom regional distributions of endophytic microbiota exist across large geographical scales (different regions), but also reveal that the potential role of biogeography may have a crucial impact in shaping microbial within the region, when there are marked differences between production sites. It was also interesting to verify that Elvas showed the lowest endophytic diversity and highest incidence of *C. nymphaeae*, leading to speculate if the low presence of endophytic fungi contributed to the increase of this pathogenic fungus. It is known that although some *Colletotrichum* species can also exist as endophytes and even be protective of certain hosts (Christian et al., 2017; Mejía et al., 2014; Arnold et al., 2003), however, in olive, *Colletotrichum* spp. has been widely described as pathogen (Talhinhas et al., 2009; Moral et al., 2009).

A. pullulans was the OTU that most contributed to the similarities in Vidigueira. *A. pullulans* has been commonly reported as one of the most abundant fungal colonizers of phyllosphere and carposphere in different plant species and may be present as both epiphyte and endophyte (Andrews et al., 1994; Deshpande et al., 1992). In addition, this fungus has been described to exhibit antagonistic activity against several plant pathogens (Hartati et al., 2015; Turk and Cene Gostincar, 2018; Wachowska and Głowacka, 2014). Although no previous study has confirmed its antagonistic activity in olive trees against *Colletotrichum* spp., the high incidence of *A. pullulans* may be related to the low richness of this pathogenic fungus.

The cultivar Galega vulgar showed significant differences in the evenness and dominance of endophytic community, showing higher values when compared to Cobrançosa and Azeiteira. Although there are no studies on the endophytic communities in these three cultivars, Schulz and Boyle (2005) suggest that the

differences between host plants and their endophytic colonizers may be associated to the plant prevailing microhabitats, stress, host senescence and host defense responses, indicating that the degree of susceptibility (pathogenic fungi) or type of cultivar/fungi interaction (non-pathogenic fungi) may be the major drivers modulating the fungal community.

Additionally, Fang et al. (2013) also suggest that chemistry of plant and the interspecific competition among fungi can regulate endophytic community. In this study, Galega vulgar cultivar showed higher endophytic richness than cultivars Cobrançosa and Azeiteira. The fungal richness is based on a competitive interaction between the endophytic species and the olive plant, making these communities more specialized. Fungal endophytic specialization is an adaptive process that leads to a niche restriction and this biotic mechanism varies according to internal and external factors from the plant, such as host resistance, co-evolution, reproductive barriers, competition, parasitism and environmental variables (Moricca et al., 2012; Moricca and Ragazzi, 2008). In addition, endophytic fungi are usually specialized or exclusive in plant genus or species and together with the other fungi, form a distinct community on the plant, as it has already been observed in olive (Fisher et al., 1992; Gomes et al., 2018; Martins et al., 2016) and other trees (Cohen, 2004; Moricca et al., 2012). More recently some studies have demonstrated that endophytic richness differs between olive cultivars (Gomes et al., 2018; Martins et al., 2016). Therefore, the characteristics that influence olive cultivars at small scale may have an impact on the differences between sites. Contrary to this, some authors suggest that in the same olive cultivars the endophytic communities cannot reflect habitat variability even when they have a higher horizontal patchiness in macro-scale (e.g. hundreds kilometres scale) (Muzzalupo et al., 2014) and different soil and climatic characteristics (e.g. different regions).

In conclusion, the present study provides a comprehensive picture of the spatial and temporal distribution of the endophytic richness and diversity in the phyllosphere of different olive cultivars. The results described here demonstrate that changes in season, site and cultivar shape the endophytic fungi, and reinforce the significance of exploring fungal biodiversity in olive cultivars. The existent information was very limited regarding the isolation and characterization of endophytes from important Portuguese cultivars such as Galega vulgar, Cobrançosa and Azeiteira and on their spatial and temporal distributions, and results here presented give an important contribution to this field. The olive fungal community was found to contain known benefic and phytopathogenic microorganisms that can have a significant impact on olive production. Beneficial endophyte colonizers, may be further explored as antagonists of important olive pathogens, and possibly be developed as effective biocontrol agents.

Acknowledgments

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