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Estimation and Diversity of Phylloplane Mycobiota on Selected Plants in a Mediterranean–Type Ecosystem in Portugal

J. Inácio,[1] P. Pereira,[2] M. de Carvalho,[2] Á. Fonseca,[1] M.T. Amaral-Collaço,[2] I. Spencer-Martins[1]

[1] Centro de Recursos Microbiológicos (CREM), Biotechnology Unit, Faculty of Sciences and Technology, Universidade Nova de Lisboa, Caparica, Portugal
[2] Department of Biotechnology, Instituto Nacional de Engenharia e Tecnologia Industrial (INETI), Lisbon, Portugal

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ABSTRACT

Mediterranean ecosystems have not been consistently investigated as natural habitats for microbes in general, and fungi in particular. Here we present the results of a survey of epiphytic mycobiota (filamentous fungi and yeasts) on the phylloplane of selected plants in the Arrábida Natural Park, an ecosystem of Mediterranean characteristics in Portugal, using conventional culture-dependent isolation methods. Leaves from the species *Acer monspessulanum* and *Quercus faginea* (deciduous trees) and *Cistus albidus, Pistacia lentiscus*, and *Osyris quadripartita* (evergreen shrubs) were collected twice a year for two consecutive years, at two distinct locations of Serra da Arrábida: the more humid northern slope and the drier southern slope. A total of 1029 strains of filamentous fungi and 540 strains of yeasts were isolated, which represented at least 36 and 46 distinct species, respectively. Total counts were higher on the plants from the northern slope and there was a general increase from spring to autumn, notably on the deciduous trees for the yeasts. Plant species that had higher numbers of leaf colonists (*A. monspessulanum, C. albidus*, and *Q. faginea*) also yielded a wider range of species. Among the filamentous fungi there was a predominance of species of ascomycetous affinity, whereas basidiomycetous species dominated among yeast isolates. Some of the taxa recovered were common to other phylloplane studies (e.g., ubiquitous molds and yeasts such as *Cladosporium* spp. and *Cryptococcus* spp., respectively), but less common species were also found, some of which appeared to represent undescribed taxa. Interestingly, a few species seemed to be associated with a particular plant, notably in the case of the evergreen shrub *C. albidus*. However, for a considerable number of fungi and yeasts the same taxon was recovered throughout the year from more than one plant and at both sites, suggesting that such species might be genuine phylloplane inhabitants (or at least of aerial plant surfaces) even though they appeared not to display host specificity.

Correspondence to: Á. Fonseca; E-mail: amrf@fct.unl.pt

Introduction

The surface of plant leaves, usually referred to as the phylloplane, represents an important terrestrial habitat and harbors a wide range of microorganisms [3 and references therein]. Fungi (encompassing both filamentous and yeast taxa) constitute a major component of the phylloplane microbiota [2, 6, 11, 12, 16, 27], but most investigations on their occurrence in this type of habitat have focused mainly on the inventory and description of new or specific taxa, generally in a restricted number of ecosystems and in some cases with emphasis on phytopathogenic taxa [1, 7, 9, 13, 18, 20, 28, 38]. Both the surface and the interior of leaves can be colonized by fungi with varied consequences for the host plants [23]. The epiphytic fungi, the surface inhabitants, depend on a thin film of nutrients that are deposited on the leaf from the atmosphere (e.g., contained in pollen grains) by insects and other organisms or that are exuded from the leaf itself [21, 22, 41]. As for other microbes, the concentration of fungi on the phylloplane is influenced by factors such as nutrient availability, humidity, leaf age and type, presence of inhibitors, immigration (arrival of viable propagules on the phylloplane), and emigration (removal or physical loss of viable propagules) [3, 22].

In the case of filamentous fungi growth, on leaf surfaces is generally enhanced when moisture levels are high and temperatures are moderate [7, 13, 22]. These microorganisms have been found on leaf surfaces of many plant species in both temperate and tropical ecosystems [6, 7, 11, 12, 18, 23, 32]. In general, there appears to be a marked dominance of deuteromycetes, mostly of ascomycetous affinity. The most common genera found on the phylloplane are *Cladosporium*, *Aspergillus*, *Alternaria*, *Aureobasidium*, and *Epicoccum* [1, 6, 7, 16, 23]. Only occasional colonies of either basidiomycetous or zygomycetous microfungi have been reported [6, 14, 20]. Yeasts are also found on the phylloplane of different plant species in both temperate and tropical regions [27, 35, 38]. As observed in the case of other aerial plant surfaces [8, 28], there appears to be a marked dominance of basidiomycetous yeasts on the phylloplane, namely of species belonging to the genera *Sporobolomyces*, *Rhodotorula* (collectively referred to as the "pink yeasts"), and *Cryptococcus* ("white yeasts") [30, 35]. Moreover, members of the genera *Bullera, Sporobolomyces*, and *Tilletiopsis* are thought to be especially adapted to this kind of environment, because of the production of forcibly ejected ballistoconidia, and are thus commonly isolated from leaves [e.g., [33]]. In a few cases, however, ascomycetous yeasts, which frequently dominate the mycobiota of flowers and fruits [e.g., 35], have been reported as important leaf colonists [31, 35].

Serra da Arrábida is a small chain of limestone outcrops with a maximum elevation of 500 m running along an East-West direction, parallel to the Southern edge of the Setúbal peninsula and falling as steep cliffs into the sea [10, 34]. It encompasses different areas with specific formations of typical Mediterranean vegetation of great interest, which has led to the creation of the Arrábida Natural Park. The predominant plant cover consists of forests and shrub formations of sclerophyll species such as *Quercus coccifera, Phyllirea latifolia, Arbutus unedo*, and *Pistacia lentiscus* (additional information on the bioclimatic characteristics and on the vegetation and flora of Serra da Arrábida can be found in [10] and [34]). Plants in Mediterranean ecosystems have not been consistently investigated as natural habitats for microbes in general, and fungi in particular, and nothing is known on the possible association between members of the phylloplane mycobiota and the prevalent plant species. The present work aimed at a preliminary evaluation of the abundance, diversity, and seasonal variation of epiphytic mycobiota (filamentous fungi and yeasts) on the phylloplane of selected plant species of the Arrábida Natural Park. We chose five native Mediterranean species representative of the Arrábida plant cover [10, 34]—two deciduous trees, *Acer monspessulanum* and *Quercus faginea*, and three evergreen shrubs, *Cistus albidus, Pistacia lentiscus*, and *Osyris quadripartita*—and two sampling sites with distinct climatic conditions (see Methods). This approach was intended to provide clues on the repercussion of the different variables—plant species, plant type (deciduous vs evergreen), location, and humidity levels—on the densities and diversity of the fungal populations. The two annual collections (spring and autumn) that were carried out further allowed us to evaluate possible variations in fungal diversity and population size throughout the year, expected to be more evident for the deciduous plants. We used culture-dependent isolation methods, which despite their well-known limitations, such as the selective nature of conventional culture media [e.g., 3], are expected to provide a first insight into the nature and abundance of phylloplane mycobiota on Mediterranean plants in the investigated area. The results obtained may in turn give forth valuable clues for additional studies on the microbial ecology of this type of ecosystem.

Methods

Study Area

Serra da Arrábida (38°27′ N, 9°02′ W) is generally characterized by an Atlantic-Mediterranean climate and consists of different microclimatic areas determined by the varying orientation of the landscape and orography [34]. Two study locations were selected: Fonte do Veado (38°28′50″ N, 9°0′17″ W; 300 m elevation), a humid site with more pronounced Atlantic influence located on the northern slope and consisting of deciduous oak and *Acer* woodland, and Mata do Solitário (38°27′55″ N, 8°59′35″ W; 50 m elevation), subhumid to semiarid site on the southern slope consisting of mixed sclerophyll and deciduous woodland and ''maquis'' formations (for additional details on the climate and vegetation of Arrábida see [10] and [34]). Leaf samples were collected from five plant species: *Quercus faginea*, *Pistacia lentiscus*, and *Cistus albidus*, present in both locations, and *Acer monspessulanum* and *Osyris quadripartita*, which are specific to Fonte do Veado and Mata do Solitário, respectively.

Sample Collection

Collections took place in early spring (March) and in mid-autumn (November), during two consecutive years (March 1997 to November 1998, for filamentous fungi, and November 1997 to March 1999, for yeasts; see Fig. 1), and at both selected sites. At each sampling date healthy leaves were collected from the same individual plants of each species, which were located in an area with a radius of 100–200 m. To avoid possible distortions in counts due to local fluctuations in fungal populations, leaves were chosen at random from distinct areas of the tree or shrub. The leaves were picked with sterile forceps and placed in sterile polyethylene bags, which were kept in a cool container (for a period that, in principle, did not exceed 4–5 h) until they were processed in the laboratory. In a few cases leaves were stored at 4°C, in the laboratory, for no longer than 24 h.

Isolation

The isolation of both filamentous fungi and yeasts was based on the plating of leaf washings. In the case of filamentous fungi, a preliminary washing step was carried out in order to avoid plating of phyllosphere conidia deposited on the leaf surfaces [1]. For this purpose, 5 g of leaves from each plant was washed by mechanical shaking in 100 mL of sterile distilled water. The water was decanted and the washing step repeated six times. The following procedure was then used for the isolation of molds and yeasts. Leaves were cut to ca 10 × 10 mm sized pieces with a sterile scalpel and 1 g from each plant was suspended in 10 mL of sterile Ringer's solution (NaCl 0.45% w/v), followed by vigorous shaking for 1 min. This procedure, followed in previous phylloplane studies [11, 20, 30, 32], may yield inocula from endophytic mycobiota, but the latter are probably outnumbered by epiphytic cells and should not be significant. The supernatant was diluted 10- to 10^4-fold and duplicate aliquots of 0.1–0.2 mL from each suspension were spread onto plates containing the following media: glucose 1% (w/v); mycological peptone 0.5%; dipotassium phosphate 0.1%; magnesium sulfate 0.05%; rose Bengal 0.005%; agar 1.6% (mold medium) or malt extract 0.7% (w/v), Soytone 0.25%, yeast extract 0.05%, rose Bengal 0.004%, agar 1.5% (yeast medium). Both media were supplemented with chloramphenicol (0.01–0.05% w/v) to prevent bacterial growth. The plates were incubated in the dark, at 20–25°C, and colonies were counted after 3, 5, and 7 days and expressed as colony-forming units (CFU) cm^{-2} leaf area.

At least one colony from each macromorphological type was picked for purification on the following media: malt extract 3% (w/v); mycological peptone 0.5%; agar 1.5% (MEA medium) for molds and the "yeast medium" without rose Bengal or chloramphenicol for yeasts (MYP medium). Isolates were maintained on slants of the latter media at 4°C. Means and standard deviations were calculated for each sample (see Fig. 1). Statistical analysis employed single classification analysis of variance (using a significance level of 5%) [40]. Densities for the same season were combined when the observed values were concordant in consecutive years for the same plant.

Identification of Isolates

Identification of filamentous fungi followed standard methods [14, 36], which are mostly based on macro and micro-morphological features such as colony diameter, texture, color, and the dimensions and morphology of hyphae and of reproductive structures (when present). In some cases, physiological features were also determined, such as growth temperatures and assimilation tests of specific carbon and nitrogen compounds. Nonsporulating isolates (i.e., molds without any kind of spores or other differentiated structures) that could not be assigned to any taxonomic group will be referred to as sterile mycelia. Yeast identification followed the standard morphological and physiological tests, as described by Yarrow [42], and the dichotomic keys presented in Kurtzman and Fell [25] and Barnett et al. [4]. For selected strains that could not be clearly identified by the previous methods (ca. 15% of the total number of yeast isolates) we determined nucleotide sequences from the D1/D2 domain of the 26S ribosomal RNA gene, a region that is being used successfully for the molecular identification of yeasts [e.g., 15]. DNA isolation and PCR amplification for sequencing were performed as described by Sampaio et al. [39]. DNA amplification used universal fungal primers ITS 5 (5′ GGA AGT AAA AGT CGT AAC AAG G) and LR6 (5′CGC CAG TTC TGC TTA CC). The sequence from the D1/D2 600–650 base pair region at the 5′ end of the 26S rDNA was obtained with an ALFexpressII Automated Sequencer (Amersham-Pharmacia). Cycle sequencing employed forward primer NL1 (5′ GCA TAT CAA TAA GCG GAG GAA AAG) and reverse primer NL4 (5′ GGT CCG TGT TTC AAG ACG G). The nucleotide sequences obtained were checked against the sequences for all currently recognized yeast species available in

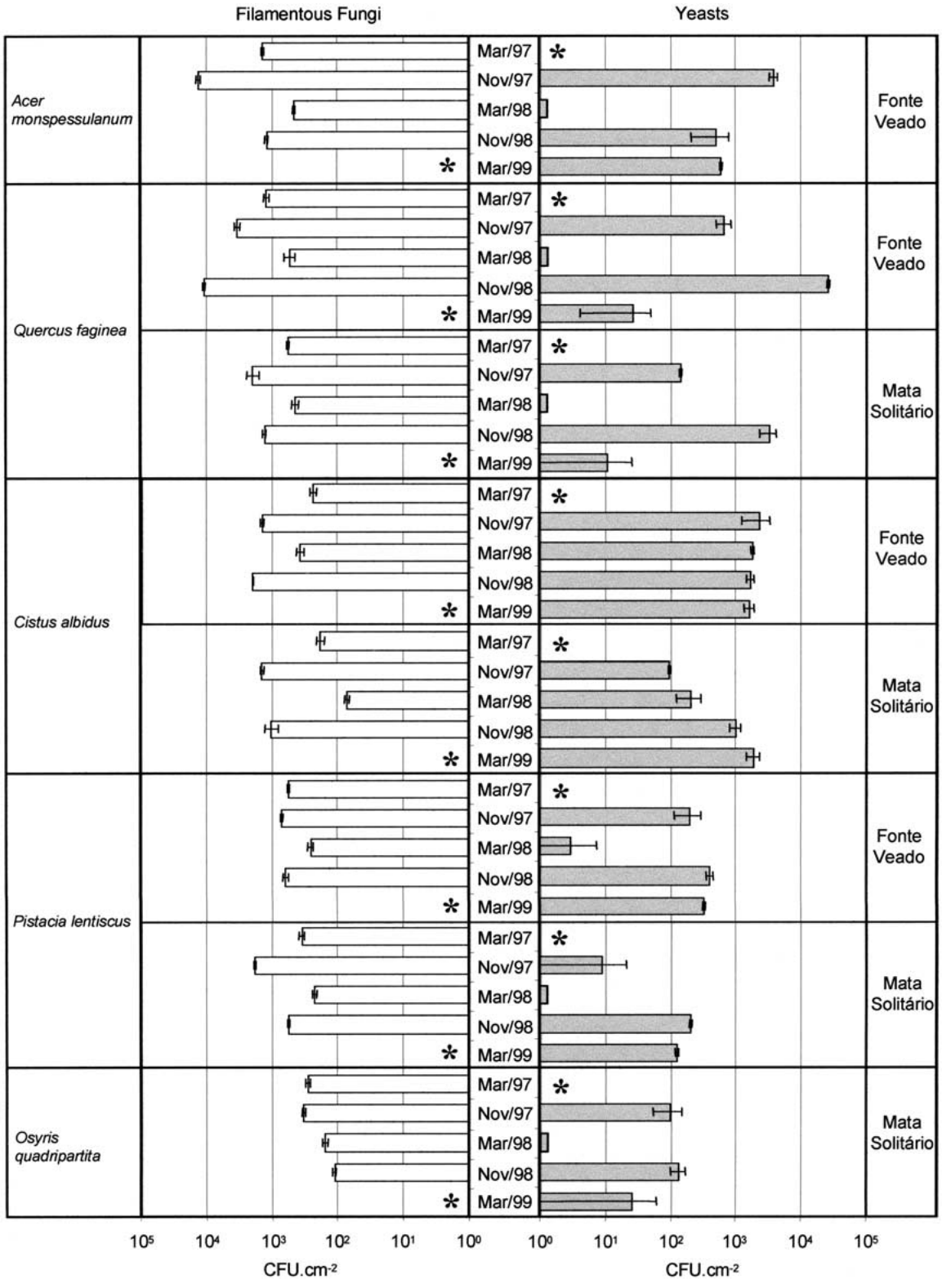
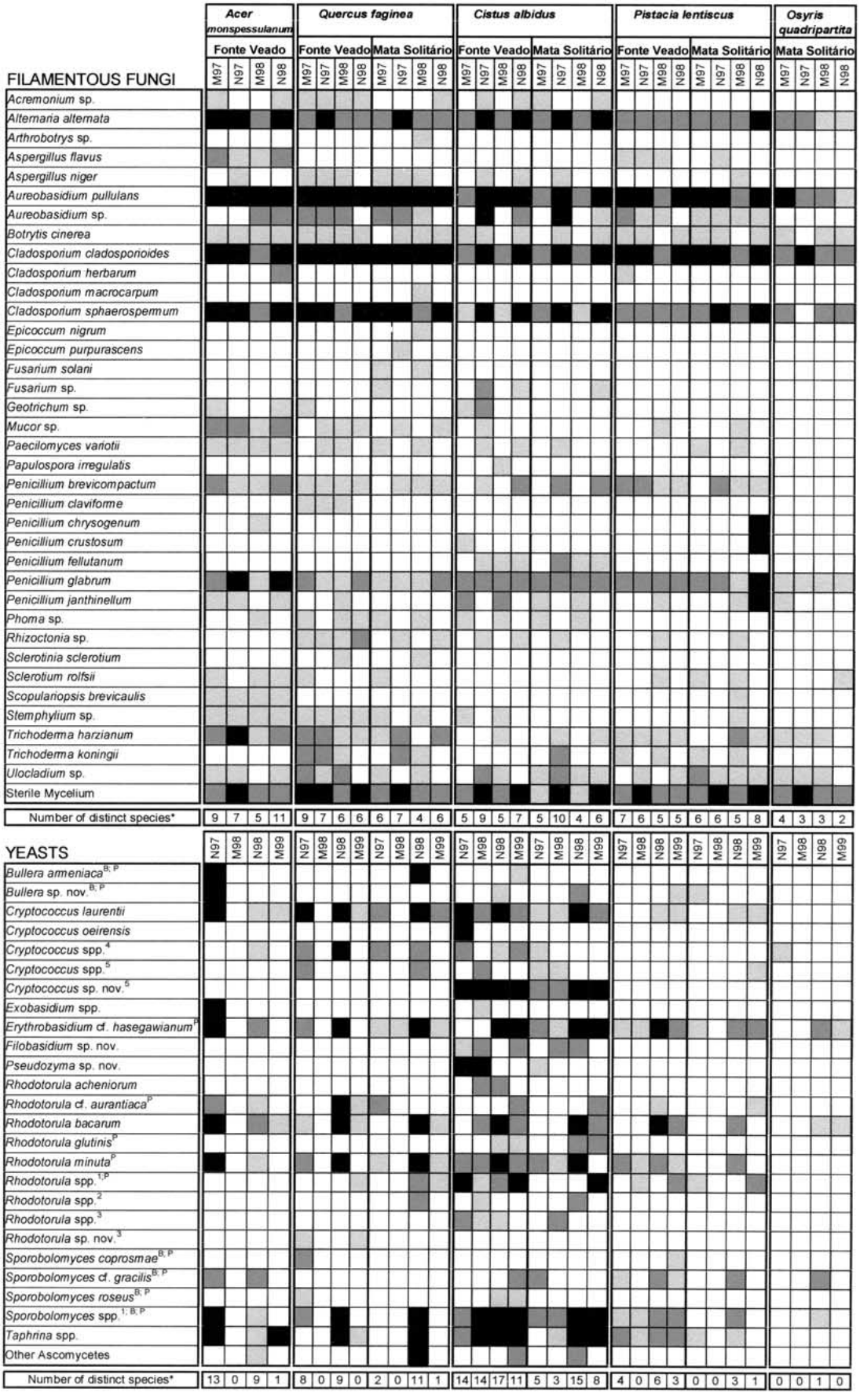


Fig. 1. Total counts of filamentous fungi and yeasts on the phylloplane of the different plant species for each sampling date, at the two sites (Fonte do Veado or Mata do Solitário); CFU, Colony-forming units; error bars represent the standard deviation of mean densities; *, not sampled.


Acer monspessulanum
Quercus faginea
Cistus albidus
Pistacia lentiscus
Osyris quadripartita
Fonte Veado
Mata Solitário
M97 N97 M98 N98 M99
FILAMENTOUS FUNGI
Acremonium sp.
Alternaria alternata
Arthrobotrys sp.
Aspergillus flavus
Aspergillus niger
Aureobasidium pullulans
Aureobasidium sp.
Botrytis cinerea
Cladosporium cladosporioides
Cladosporium herbarum
Cladosporium macrocarpum
Cladosporium sphaerospermum
Epicoccum nigrum
Epicoccum purpurascens
Fusarium solani
Fusarium sp.
Geotrichum sp.
Mucor sp.
Paecilomyces variotii
Papulospora irregulatis
Penicillium brevicompactum
Penicillium claviforme
Penicillium chrysogenum
Penicillium crustosum
Penicillium fellutanum
Penicillium glabrum
Penicillium janthinellum
Phoma sp.
Rhizoctonia sp.
Sclerotinia sclerotium
Sclerotium rolfsii
Scopulariopsis brevicaulis
Stemphylium sp.
Trichoderma harzianum
Trichoderma koningii
Ulocladium sp.
Sterile Mycelium
Number of distinct species*
9 7 5 11 9 7 6 6 6 7 4 6 5 9 5 7 5 10 4 6 7 6 5 5 6 6 5 8 4 3 3 2
YEASTS
Bullera armeniaca[B; P]
Bullera sp. nov.[B; P]
Cryptococcus laurentii
Cryptococcus oeirensis
Cryptococcus spp.[4]
Cryptococcus spp.[5]
Cryptococcus sp. nov.[5]
Exobasidium spp.
Erythrobasidium cf. hasegawianum[P]
Filobasidium sp. nov.
Pseudozyma sp. nov.
Rhodotorula acheniorum
Rhodotorula cf. aurantiaca[P]
Rhodotorula bacarum
Rhodotorula glutinis[P]
Rhodotorula minuta[P]
Rhodotorula spp.[1; P]
Rhodotorula spp.[2]
Rhodotorula spp.[3]
Rhodotorula sp. nov.[3]
Sporobolomyces coprosmae[B; P]
Sporobolomyces cf. gracilis[B; P]
Sporobolomyces roseus[B; P]
Sporobolomyces spp.[1; B; P]
Taphrina spp.
Other Ascomycetes
Number of distinct species*
13 0 9 1 8 0 9 0 2 0 11 1 14 14 17 11 5 3 15 8 4 0 6 3 0 0 3 1 0 0 1 0

public databases (GenBank). Sequences obtained in this study are available upon request to the authors.

Results and Discussion

Total Mycobiota

The results presented in Figs. 1 and 2 show that the phylloplane of the sampled plants is colonized by considerably large and diverse fungal populations. Total counts and species richness varied according to plant species, season, and sampling site. Relative frequencies of molds and yeasts were generally concordant and combined totals ranged from ca 1×10^2 CFU cm^{-2} on *O. quadripartita* to values well above 1×10^4 CFU cm^{-2} on *A. monspessulanum* and *Q. faginea* (Fig. 1). In a recent study on the phylloplane of mango trees in South Africa using a similar isolation method [11], mold and yeast densities were in the range 1×10^4 to 1×10^5 CFU cm^{-2} (although *Aureobasidium pullulans* was counted as a member of the yeast population). In the present study and for the plant species that occurred at both sampling sites, the total number of isolates of either filamentous fungi or yeasts were generally higher on the samples from Fonte do Veado, regardless of season (Figs. 1 and 2). The differences are statistically supported for filamentous fungi on *C. albidus* (spring, $p = 0.002$, and autumn, $p = 0.020$) and *Q. faginea* (spring, $p = 0.037$, and autumn, $p = 0.008$) and for yeasts on *C. albidus* (spring and autumn, $p = 0.007$). This result might be explained by the more humid conditions that prevail on the northern slope throughout the year, which concurs with similar observations about the effect of high humidity levels on the density of phylloplane microbial populations made by other authors [e.g., 5, 22]. On the other hand, there was a general increase in the number of isolates on the leaves of the deciduous tree species (*A. monspessulanum* and *Q. faginea*) from spring to autumn (Fig. 1). The differences are statistically supported for filamentous fungi: on *A. monspessulanum* ($p = 0.040$) and on *Q. faginea* (Fonte do Veado, $p = 0.004$; Mata do Solitário, $p < 0.001$). This trend was confirmed in the case of the yeasts by the results of an extra sampling in the summer of 1998, which yielded intermediate numbers of leaf colonists between the spring and autumn values (data not shown). This observation is in agreement with the prevailing view that phylloplane fungal populations on deciduous plants increase with leaf development reaching a peak at mid-season or at leaf-fall [7, 17, 20, 27]. It is conceivable that on deciduous trees the colonizers of newly opened leaves originate from cells that overwintered in the buds or on the bark; they may also result from air- or insect-borne immigrants present on the evergreens, namely in the case of the widespread species that were found on more than one plant at both sites (Fig. 2). This seasonal trend was not so evident on the evergreens (Fig. 1), except for the filamentous fungi on *C. albidus* at both sites ($p < 0.001$). However, counts were consistently low in the case of the yeasts on *P. lentiscus* and *O. quadripartita* (Fig. 1). The latter are both xerophytes producing large amounts of terpenoid compounds that might exert a selective effect on the potential leaf colonizers [e.g., 29]. On the phylloplane of evergreens, the inoculum for young leaves is most probably provided by cells that are washed out or blown out of older leaves on the same plant and therefore total counts would be expected to remain constant throughout the year. This appears to be the case for the yeasts on *C. albidus* where the differences between spring and autumn are not statistically significant (Fonte do Veado, $p = 0.484$; Mata do Solitário, $p = 0.421$).

It is worth noting that total counts and species richness for both filamentous fungi and yeasts were not significantly different when comparing the results of the autumn collections for the deciduous trees with those of the evergreens (excluding *O. quadripartita*) (Figs. 1 and 2). It could be anticipated that phylloplane populations on the evergreens would be more numerous because of the longevity of their leaves [e.g., 11], but in some cases the opposite was observed (Fig. 1), e.g., the combined densities of filamentous fungi for the autumn samplings of the deciduous trees (*A. monspessulanum* and *Q. faginea*) at Fonte do Veado were statistically higher ($p < 0.001$) than the corresponding values for the evergreens (*Cistus albidus* and *Pistacia lentiscus*). These results suggest that in this particular case populations of mycobiota become established on the phylloplane of the deciduous trees in a

Fig. 2. Species diversity and relative frequency of occurrence of filamentous fungi and yeasts on the phylloplane of the different plant species, according to sampling site and date (M, March; N, November). Shade code: black, $>10^2$ CFU cm^{-2}; dark gray, 10^1–10^2 CFU cm^{-2}; light gray, $<10^1$ CFU cm^{-2}; white, not detected. [B]Ballistoconidia-forming yeasts; [P]pigmented yeasts. Phylogenetic affiliation of yeasts: [1]Erythrobasidium clade, [2]Ustilaginales, [3]Microbotryum clade, [4]Filobasidiales, [5]Tremellales. *Only species with densities $>10^1$ CFU/cm^{-2} were considered and some entries include more than one distinct species, e.g., *Cryptococcus* spp.

relatively short period (from spring to autumn) and that leaf turnover, nutrient availability, or other intrinsic factors (e.g., presence of phytochemicals with antimicrobial activity on the leaves of evergreens—see above) effect some control on the populations levels on the evergreens.

Analysis of Fig. 2 shows that plant species having higher numbers of leaf mycobiota (*A. monspessulanum, C. albidus*, and *Q. faginea*) also yielded a slightly wider range of species (Fig. 2). The results further suggest that the most prevalent mold or yeast species followed the overall seasonal trend, their numbers rising from spring to autumn, and therefore they may correspond to the most representative phylloplane inhabitants (see below). These taxa are, in most cases, not restricted to a particular plant, but a few examples emerged of specific associations (see below). Other less frequent species may represent occasional colonists that originate from cells arising from the air, deposited by insects or rain-washed from other aerial plant parts. Testing of these hypotheses would, however, require additional isolations from all possible sources.

Filamentous Fungi

The total numbers of filamentous fungi recovered from each sample is shown in Fig. 1, with values ranging from ca 10^2 to 10^4 CFU cm^{-2}, and were generally higher than for the yeasts. As pointed out above, molds were apparently more numerous on plant leaves from Fonte do Veado than from Mata do Solitário (Fig. 1), except for *C. albidus* and *P. lentiscus* in November 1997, the latter showing a significantly larger number (ca 2.5-fold) of isolates from Mata do Solitário. As already mentioned, there was an increase in the number of isolates from spring to autumn on the deciduous trees and, in the case of filamentous fungi, also on the evergreens *C. albidus* and *P. lentiscus* (Fig. 1). *Q. faginea* and *A. monspessulanum* consistently had the highest number of leaf colonists in both seasons, with pronounced peaks in the November collections.

The majority of isolates were deuteromycetes, mostly of ascomycetous affinity (77% of a total of 1029 isolates). Another considerable fraction of the total number of isolates (19%) were nonsporulating species and were thus included in the sterile mycelia group. This observation is in agreement with the results of other phylloplane studies [1, 6, 20]. Isolates that could be identified represented at least 36 distinct species (10 of which were only identified to the genus level) and were distributed as shown in Fig. 2. Species that were recovered from all samples included *Cladosporium cladosporioides, Aureobasidium pullulans, Alternaria alternata*, and *Penicillium glabrum*. Fungi belonging to the sterile mycelia group were also found in all samples with frequencies ranging from 10 to 10^3 CFU cm^{-2}, but on *A. monspessulanum* and *Q. faginea* leaves from Fonte doVeado this heterogeneous group of fungi appeared with frequencies higher than 10^3 CFU cm^{-2}. A few fungal species that have been reported by others as common inhabitants of the phylloplane, such as *Cladosporium sphaerospermum, Trichoderma harzianum, Penicillium brevicompactum*, and *Aureobasidium* spp. [6, 7, 12], were also found to occur with high frequencies in the plants studied in the present work. Overall, *Q. faginea* was the plant that showed the highest degree of colonization and species richness (Fig. 2), followed by *A. monspessulanum*. Some fungi appear to be restricted to *Q. faginea*, namely *Penicillium claviforme, Cladosporium macrocarpum, Fusarium solani, Epicoccum purpurascens, Epicocum nigrum*, and *Arthrobotrys* sp. However, these species were found at low frequencies and only occasionally. *Penicillium fellutanum* appears to be restricted to the leaves of *C. albidus* from both locations and *Scopulariopsis brevicaulis* was consistently isolated from *A. monspessulanum*. On the other hand, *O. quadripartita* yielded the narrowest range of species (Fig. 2). Some species reported as common phylloplane inhabitants, such as *Cladosporium herbarum* [1, 14, 20], *E. purpurascens, E. nigrum* [1, 7, 9, 14, 16], *Papulospora irregularis*, and *Cheaetomium* sp. [14], were only rarely found in the present study. Moreover, typical soil fungi, e.g., Mucorales, *Aspergillus, Trichoderma, Scopulariopsis*, and *Paecilomyces* spp., which appear to be active in leaf litter decomposition [19, 37] and are often recovered from leaf surfaces [18, 20, 26], were a relatively minor component of the total isolates in our study.

Direct comparison of the data of the present study with those obtained by other authors is hampered by differences in isolation methods and frequency of sampling. However, it appears that fungal species richness on phylloplane samples from Serra da Arrábida (36 distinct species) is comparatively lower than the values reported for fungal communities from other ecosystems, especially tropical and temperate habitats [1, 6, 7, 12, 18]. For example, from the phylloplane of four trees in a primary rain forest of Costa Rica an average of 134 distinct fungal species were isolated and identified [6]. A survey of the evergreen shrub qat (*Catha edulisis*) located in the "coffee-

zone" provinces of Yemen Arab Republic, yielded 64 fungal species [1], whereas ca 53 distinct fungal species were collected from leaves of *Acer platanoides* collected in the grounds of the University of Stirling, Scotland [7].

Yeasts

To our knowledge, the only information on the yeast biota associated with plant species in a geographical area with Mediterranean characteristics was recently provided by Middelhoven [31]. Samples were collected in the Canary Islands, from plants we have not investigated, and the few yeasts recovered included representatives of the genera *Debaryomyces, Cryptococcus*, and *Rhodotorula*. In the present work, the vast majority of isolates were of basidiomycetous affinity (ca 93% of a total of 540 strains), in agreement with the results of other phylloplane studies. Most basidiomycetous isolates were found to belong to the following major phylogenetic clades *sensu* Fell et al. [15]: Tremellales, Filobasidiales, Ustilaginales, Microstromatales, Microbotryum, and Erythrobasidium (Fig. 2). They represented at least 45 species (note that some entries in Fig. 2 include more than one distinct species under a single designation: e.g., *Cryptococcus* spp.). Several yeasts were present in all leaves regardless of plant or location: apart from species that are known to be ubiquitous on leaves, such as *Cryptococcus laurentii, Rhodotorula minuta*, and *Sporobolomyces* spp., we recovered significant numbers of strains of *Rhodotorula bacarum* and of species closely related to *Erythrobasidium* cf. *hasegawianum* and *Rhodotorula* cf. *aurantiaca*, which have not been commonly isolated from the phylloplane [27, 35]. Some of these species were present in high densities that followed the seasonal trend observed for the total yeast population (e.g., *Rhodotorula minuta*—Figs. 1 and 2). A surprising result was the low incidence of ballistoconidia-forming yeasts, which could be attributed either to their lower relative concentration or to a stronger attachment of these yeasts to the leaf surfaces preventing their recovery by the isolation procedure employed in this study. However, using an isolation method specific for those types of cells (the ballistospore-fall method) we were able to isolate many strains, mainly of *Sporobolomyces roseus* and *Tilletiopsis* spp. (data not shown). The latter method is, however, not amenable to quantification. Among the few ascomycetous isolates, we found significant numbers of presumptive yeast stages of *Taphrina* spp., which were present in a large number of samples from all plants (except *O. quadripartita*) (Fig. 2). This finding was somewhat surprising, since the yeast stages of these dimorphic phytopathogenic fungi are rarely isolated from natural substrates except from infected plant material [e.g., 24].

Previous studies have pointed to an apparent lack of specificity of the basidiomycetous yeasts that occur on the phylloplane, namely *Cryptococcus* spp. and the ballistoconidia-producing species *Sporobolomyces roseus* and *Bullera alba* [27], a hypothesis partly confirmed in the present study. However, some species appear to be restricted to *Cistus albidus*, namely *Rhodotorula acheniorum* and three putative novel species: a *Filobasidium* sp. (Filobasidiales), *Pseudozyma* sp. (Ustilaginales), and, in exceptionally high densities, a *Cryptococcus* sp. resembling *Cryptococcus hungaricus* (Tremellales). The yeast abundance and species richness on the phylloplane of this plant is particularly conspicuous and may be a consequence of the dense trichome cover of its leaves [3].

The quantitative results presented in Fig. 1 are difficult to compare to those from previous reports because, in some of these studies, the ubiquitous dimorphic deuteromycete *Aureobasidium pullulans* is considered as part of the yeast population [7, 11]. However, frequencies ranging up to 10^4 CFU cm^{-2}, such as the ones we obtained, are not uncommon [e.g., 27]. The observed frequencies were conspicuously low on *P. lentiscus* and, especially, on *O. quadripartita*. On these two plants, we observed a higher proportion of pigmented yeast species than on the other plants (Fig. 2). A possible selective advantage of pigmented yeasts on these xerophytes warrants further study. The seasonal variation of total yeast counts observed on the deciduous trees as compared to the evergreen shrubs was more pronounced than that observed for the filamentous fungi (Fig. 1).

Conclusion

To our knowledge, no information is so far available on the number, or diversity, of either filamentous fungi or yeasts associated with the plant species sampled in the present study. In spite of the known limitations of the isolation methods employed and of some apparent overlap with results of previous phylloplane surveys, both in quantitative and qualitative terms, our work is thought to constitute an important contribution to the assessment of microbial diversity in a Mediterranean-type ecosystem. It is worth noting that a number of isolates appeared to

represent undescribed species. This seems to be the case of ca 25 distinct yeast species (corresponding to 23% of the total number of isolates); however, 10 of these were represented by single strains. Molecular identification of molds that would allow similar calculations for these fungi is currently underway. Formal descriptions of putative novel taxa are, however, beyond the scope of this report and will be presented elsewhere. It is nevertheless clear that our knowledge about phylloplane mycobiota is still scarce and that more surveys are needed for the ecological characterization of microbial populations on this environment. Finally, to unveil new species and reveal specific plant–microbe associations holds a considerable biotechnological potential, e.g., the use of fungi as biological control agents [8, 28] or for the production of novel antimicrobial compounds [30].

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