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Fungal endophytic communities associated to the phyllosphere of grapevine cultivars under different types of management

Carla Marisa Reis VARANDA[a],*, Mônica OLIVEIRA[a], Patrick MATERATSKI[b], Miguel LANDUM[a], Maria Ivone Esteves CLARA[c], Maria do Rosário FÉLIX[c]

[a]ICAAM — Instituto de Ciências Agrárias e Ambientais Mediterrânicas, Instituto de Investigação e Formação Avançada, Universidade de Évora, Núcleo da Mitra, Ap. 94, 7006-554 Évora, Portugal
[b]MARE — Centro de Ciências do Mar e do Ambiente, Universidade de Évora, Escola de Ciências e Tecnologia, c/o NemaLab, Apartado 94, 7002-554 Évora, Portugal
[c]Departamento de Fitotecnia, Instituto de Ciências Agrárias e Ambientais Mediterrânicas, Instituto de Investigação e Formação Avançada, Universidade de Évora, Núcleo da Mitra, Ap. 94, 7006-554 Évora, Portugal

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ABSTRACT

Fungal endophytes present in different asymptomatic grapevine plants (*Vitis vinifera* L.) located in different vineyards within Alentejo, a highly important viticulture region in Portugal, were identified in this study. Sampled grapevine plants included the three most representative cultivars in the region, Syrah, Cabernet Sauvignon, and Aragonez, growing under two different modes of management, conventional and biological. Sixteen fungal taxa were identified through sequencing of the internal transcribed spacer region. Total number of endophytic fungi isolated showed significant differences both in management mode and in cultivars, with higher numbers in grapevines under conventional mode and from Syrah cultivar. The composition of fungal endophytic communities did not show significant differences among cultivars, but differences were observed between fungal communities isolated from grapevines under biological or conventional modes. The most fungal taxa isolated from grapevines cultivated under biological mode were *Alternaria alternata*, *Cladosporium* sp., and *Nigrospora oryzae*, and under conventional mode *Botrytis cinerea*, *Epicoccum nigrum*, and *Epicoccum* sp. These differences suggest that the different products used in grapevine production have impacts in fungal endophytic composition. Further investigation of the identified fungi with respect to their antagonistic characteristics and potential use in plant protection to ensure food safety is now in course.

Introduction

Endophytes are microorganisms that live entirely within plant tissues, roots, stems, and/or leaves, without causing any apparent symptoms of disease (Petrini 1991). Fungal endophytes are ubiquitous, they have been found within all plants from the diverse habitats examined to date (Kumaresan & Suryanarayanan 2001; Schulz *et al.* 2002; Rodriguez *et al.*

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* Corresponding author. Tel.: +351 266760851; fax: +351 266760822.
E-mail address: carlavaranda@uevora.pt (C. M. R. Varanda).

2009; Pancher *et al.* 2012). The endophytic community in a single plant is usually composed by numerous and systematically diverse species of fungi (Petrini 1991; Oono *et al.* 2015). Their number and species composition is influenced by factors such as the environment (Saona *et al.* 2010; Yousaf *et al.* 2010; Núnez-Trujillo *et al.* 2012), plant physiology (Islam *et al.* 2010), anthropogenic factors (Rasche *et al.* 2006), and pathogen infections (Araujo *et al.* 2002; Bulgari *et al.* 2011; Buyer *et al.* 2011).

Endophytes have been gaining attention in the past decade in areas such as medicine, agriculture, and industry, mostly due to the vast potential uses of their chemically diverse secondary metabolites (Tao *et al.* 2008; Rodriguez *et al.* 2009) and their roles within plants (Oono *et al.* 2015). Several studies have shown that some fungal endophytes have beneficial effects on their hosts. They may act as plant growth promoters; confer tolerance to environmental stresses and pathogen and herbivore attacks, the latter for instance by decreasing the palatability of the host tissue to herbivores through the production of toxic compounds (Arnold *et al.* 2003; Miller *et al.* 2008; Bae *et al.* 2009; Oono *et al.* 2015). The role of endophytes in pathogen defence is attained through different mechanisms, namely the induction of systemic resistance, through the accumulation of pathogenesis-related (PR) proteins, expression of plant defence genes; the production of secondary metabolites that inhibit fungal growth and the competition with pathogens for the same ecological niches in terms of nutrients and space (Arnold *et al.* 2003; Gonzalez & Tello 2011). On the other hand, endophytic fungi benefit from protection and nutrition from their hosts, and in some cases, reproducing sexually on dead tissues of their host plant (Saikkonen *et al.* 1998). This balance however may not be guaranteed in a continuous manner, either due to a decrease in plant defence or an increase in fungal virulence since some endophytes may go from a mutualistic to a parasitic lifestyle, depending on factors such as the environment, fungal community composition, host health and host-endophyte genotype—genotype interaction to name a few (Redman *et al.* 2001). This means that some pathogenic fungi may live as endophytes during part of their life, which is an interesting challenge for plant pathology to find out and understand what are the key differences for both endophytic and pathogenic lifestyles. This is particularly important in grapevine, where the agents known to cause some of the most important trunk diseases (excoriose, Petri disease, esca) have been isolated from inside plant tissues from both symptomatic and asymptomatic plants (Mostert *et al.* 2000; Halleen *et al.* 2007; Gonzalez & Tello 2011; Núnez-Trujillo *et al.* 2012).

DNA-based approaches have been largely used in grapevine mostly to identify plant pathogenic fungi (Schmidt *et al.* 2003; Oliveri *et al.* 2007; Sánchez-Torres *et al.* 2008). Endophytic fungal communities have been studied less, but have showed some very interesting and important results (Martini *et al.* 2009; Gonzalez & Tello 2011). Their presence has shown to interfere in wine quality through the production of toxic metabolites that some fungi produce, such as ochratoxin A (OTA) produced by *Aspergillus* spp. and *Penicillium* spp. (Cabanes *et al.* 2010). In addition, the accumulation of PR proteins as a result of the activation of plant defence pathways by endophytes has shown to affect wine stability (Ferreira *et al.*

2004). Some fungal endophytes in grapevine have, however, shown some beneficial effects such as antagonistic properties against some important pathogens. Fungi belonging to the genus *Alternaria* and *Epicoccum* have shown antagonism against *Plasmopara viticola* and *Botrytis cinerea* (Musetti *et al.* 2007; Polizzotto *et al.* 2009). These studies show that the study of endophytic communities in grapevine is essential both to shape future pest management and to produce high quality products. To our knowledge, in Portugal, data on such communities are inexistent.

The aim of this study was 1) to characterise the composition of fungal endophytic communities in grapevine plants in a region of relevant impact in grapevine production; 2) to find out if the composition of fungal communities is related to the type of management performed (biological and conventional); and 3) to find out if the composition of the fungal communities is related to the cultivar (Syrah, Cabernet Sauvignon, and Aragonez). Sampling was performed in two proximate locations in the south of Portugal (Estremoz and Évora).

Exploring endophytic diversity in different contexts will help to understand the variables responsible for structuring fungal diversity. It will also help to understand the role/influence that the communities have on the host, for example on grapevine terroir and wine characteristics, as well as to help to understand their relation with other pathogens or diseases.

Materials and methods

Study sites and sample collection

The sampling areas are located in Alentejo (south of Portugal), a major vine producing area, where the altitude ranges from 150 to 400 m above sea level, the climate is Mediterranean, mean temperature is 15 °C, annual rainfall is 600 mm and soils are mostly of schist and calcareous origin. Surveys were carried out during the period of 2014—2015.

A total of 12 vineyards in an area of 450.000 m^2 were sampled representing two types of management, biological or conventional, and three most produced cultivars, Syrah, Cabernet Sauvignon, and Aragonez (synonym Tempranillo) (Table 1).

The fungicide substances used in the conventional plots were fosetyl-aluminium, folpet, cymoxanil, spiroxamine, cyflufenamid, difenoconazole, copper oxychloride, chinoxifen, and myclobutanil. These substances were used to prevent excoriose, black rot, downy and powdery mildews, and Botrytis. At the time of the survey no signs of disease were observed. The fungicide substances used in the biological plots were copper hydroxide, sulphur and extracts from *Equisetum arvense* and *Saponaria officinalis*.

Ten asymptomatic plants were randomly selected in each vineyard, totalling 120 samples. Three leaves were cut from each plant and transported to the laboratory in a refrigerated basket, stored at 4 °C and processed within the next 48 h.

Isolation of endophytic fungi

Leaves were surface disinfected to suppress epiphytic microorganisms and cut into 0.5 cm^2 sections. Disinfection consisted in a succession of 3 min immersions, conducted

Table 1 − Provenience of grape leaf samples used in this study.

Type of management	Location	Cultivar	Sample
Biological	Estremoz	Syrah	BioEstSyr
		Cabernet Sauvignon	BioEstCab
		Aragonez	BioEstAra
Conventional		Syrah	ConvEstSyr
		Cabernet Sauvignon	ConvEstCab
		Aragonez	ConvEstAra
Biological	Évora	Syrah	BioEvoSyr
		Cabernet Sauvignon	BioEvoCab
		Aragonez	BioEvoAra
Conventional		Syrah	ConvEvoSyr
		Cabernet Sauvignon	ConvEvoCab
		Aragonez	ConvEvoAra

under a sterile laminar airflow chamber, in a series of 96 % ethanol, 3 % sodium hypochlorite solution, 70 % ethanol, and ultra-pure water. After disinfection, leaf pieces were dried in sterile Whatman paper, placed on Petri dishes of 9 cm diameter (four pieces per plate) containing Potato Dextrose Agar medium (PDA, Merck, Germany) and incubated, in darkness, for 1−2 weeks at 23−25 °C.

Four days later, all morphologically different colonies were isolated by transferring an about 5 mm^2 agar disk of the growing fungi to fresh medium (PDA). Mycelium from isolated colonies was ground in liquid nitrogen and stored at −80 °C for later use in DNA extraction.

Fungal DNA extraction

DNA was extracted using the CTAB (hexadecyltrimethylammonium bromide) method described by Doyle & Doyle (1987) with some modifications. Briefly, fungal DNA powder was placed in 1.5 mL microtones containing pre warmed 600 μL 2 % 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 60 min, gently mixing by inversion every 15 min; 600 μL of chloroform−isoamyl alcohol (24:1) was added to the tubes and gently mixed for 10 min. Samples were centrifuged for 10 min at 5000g, the supernatant was then transferred to a fresh tube following the addition of 2.5 volumes of cold ethanol (−20 °C). Samples were gently mixed by inversion and centrifuged at 10 000g for 20 min. The liquid solution was released and the DNA pellet washed with 500 μL of 70 % ethanol to eliminate salt residues adhered to the DNA and dried in a speed vacuum for 10 min at 55 °C. Pellet was resuspended in 50 μL of ultrapure water and stored at −20 °C. DNA concentration was determined using a Nano-Drop ND-1.000 spectrophotometer.

Fungal DNA identification

The internal transcribed spacer (ITS) region of nuclear rDNA was amplified through PCR from genomic DNA, or lysed fungal material, by using ITS1 and ITS4 primers (White et al. 1990). PCR reactions consisted of 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), 1 μM of each primer, and 2.5 U of Dream-Taq DNA polymerase (Fermentas) in a total reaction volume of 50 μL. Amplification was carried out in a Thermal Cycler (Bio-Rad) at 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 analysed by agarose gel electrophoresis. PCR products were purified using DNA Clean & Concentrator (Zymo Research) and sequenced in forward and reverse directions by Macrogen (The Netherlands). Sequence analysis of the ITS sequences was carried out using BioEdit Sequence Alignment Editor v.7.2.3 (Hall 1999). The search for homologous sequences was done using Basic Local Alignment Search Tools (BLAST) at the National Center for Biotechnology Information (NCBI). Sequences were identified to the species level whenever possible. All fungal sequences considered were at least 98 % identical to the best hit in the NCBI database.

Fungal diversity and multivariate 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. Singletons and doubletons were also determined. Several nonparametric estimators were used to infer species richness: Bootstrap, Jack 1 and Jack 2, Chao 1 and Chao 2, ACE and ICE estimators.

Diversity of endophytes was obtained for each management mode and cultivar by calculation of Simpson diversity ($D = 1/sum(Pi^2)$), Shannon−Wiener diversity ($H = −sum(Pi·ln[Pi])$), and Simpson evenness indexes ($E = H/ln[S]$), being Pi the number of a species divided by the total number of organisms observed and S the species richness.

Multivariate analyses were performed to detect significant differences in the total number of fungi present under two different types of management 'Biological' and 'Conventional' in the three cultivars, 'Syrah', 'Cabernet Sauvignon', and 'Aragonez'. The statistical analyses of the data were performed using the PRIMER v6 software package (Clarke & Warwick 2001) with the PERMANOVA add-on package (Anderson et al. 2008).

Total number of fungi was calculated using the dataset from two different types of management 'Biological' and 'Conventional' and each cultivar 'Syrah', 'Cabernet Sauvignon', and 'Aragonez'. A two-way permutational analysis of variance (PERMANOVA) was applied to test the hypothesis that significant differences existed in total number of fungi between 'Biological' and 'Conventional', among 'Syrah', 'Cabernet Sauvignon', and 'Aragonez'. The PERMANOVA analysis was carried out following the two factor design: 'Management': 'Biological' and 'Conventional' (2 levels, fixed) and 'Cultivars': 'Syrah', 'Cabernet Sauvignon' and 'Aragonez' (3 levels, random). Total fungi data were square root transformed in order to scale down the importance of highly abundant fungi genera and therefore increase the importance of the less abundant ones in analysis of similarity between communities. The PERMANOVA analysis was conducted on a Bray−Curtis similarity matrix (Clarke & 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).

A Principal Component Analysis (PCA) of presence and absence of fungal species was performed to explore patterns in multidimensional data by reducing the number of dimensions with minimal loss of information. The PCA ordination was based on each of the two different types of management 'Biological' and 'Conventional' and on each of the three cultivars 'Syrah', 'Cabernet Sauvignon', and 'Aragonez'. Prior to the calculation of the PCA ordination data were log (X + 1) transformed.

Results

Isolation and identification of endophytic fungi

In the 2-year survey presented in this work, 120 field samples were analysed and 240 endophytic fungal isolates were obtained from two vine-producing areas. Fungal isolates were obtained in all tested plants. All isolated fungi were

successfully identified based on ITS sequence analysis. Fungi were identified at species level in 40 % of the isolates. The size of the generated PCR products ranged from 500 to 700 bp.

Species diversity of endophytic fungi

The 240 isolates were identified as belonging to 16 OTUs (Fig 1) representing ten fungal genera. The species accumulation curve (Fig 2), calculated using Mao Tau algorithm, which gives confidence intervals of 95 %, indicated that the sampling efforts made were suitable to recover most of species diversity present in the phyllospheres of the plants surveyed. The actual species number was estimated to be 17 using Bootstrap estimators, 19 using Jack 1, 20 using Chao 1, 21 using ACE, ICE, Chao 2, and 22 using Jack 2, meaning that the 16 OTUs found in this study represent more than 73 % of the species richness actually present.

Nearly all isolates obtained belonged to the ascomycetes (99.6 %), only one isolate belonged to basidiomycetes (Class Agaricomycetes, Order Atheliales) (0.4 %). Within the ascomycetes, the 15 OTU represented four classes (Dothideomycetes, Leotiomycetes, Sordariomycetes, and Eurotiomycetes) being the Dothideomycetes the most representative (60 %) and the others distributed equally, with two species each. Within the Dothideomycetes, the Pleosporales were the most frequent, with seven OTUs, representing 78 % of the total Dothideomycetes, 44 % of the total OTUs and 66 % of the total isolates found.

The number of OTUs obtained from individual plants ranged from one to five and in plots from four to ten.

Most of the OTUs obtained in this study showed to be very frequent, ten (62 %) appeared in four or more plants (plurals), two (13 %) in two plants (doubletons), and four (25 %) only in one plant (singletons).

From the 240 isolates, 159 (66 %) belonged either to *Alternaria* (89 isolates) or *Epicoccum* genera (70 isolates). *Alternaria* sp. and *Epicoccum* sp. were the only OTUs detected in all vineyards. *Alternaria solani*, *Athelia* sp., *Diplodia seriata*, *Penicillium brevicompactum*, and *Penicillium* sp. were only detected in one
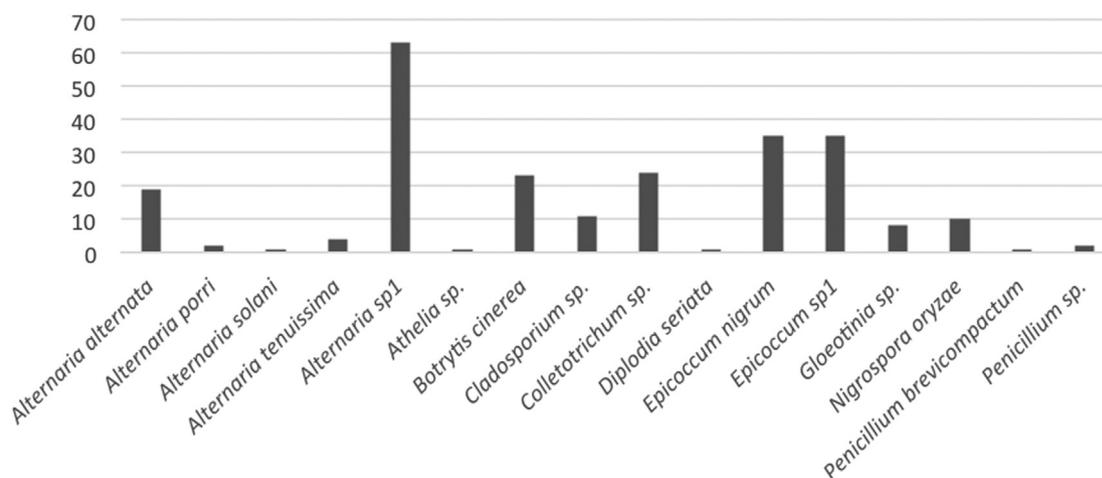
Fig 1 − **Total number of fungal isolates isolated from 12 vineyards distributed by OTU.**
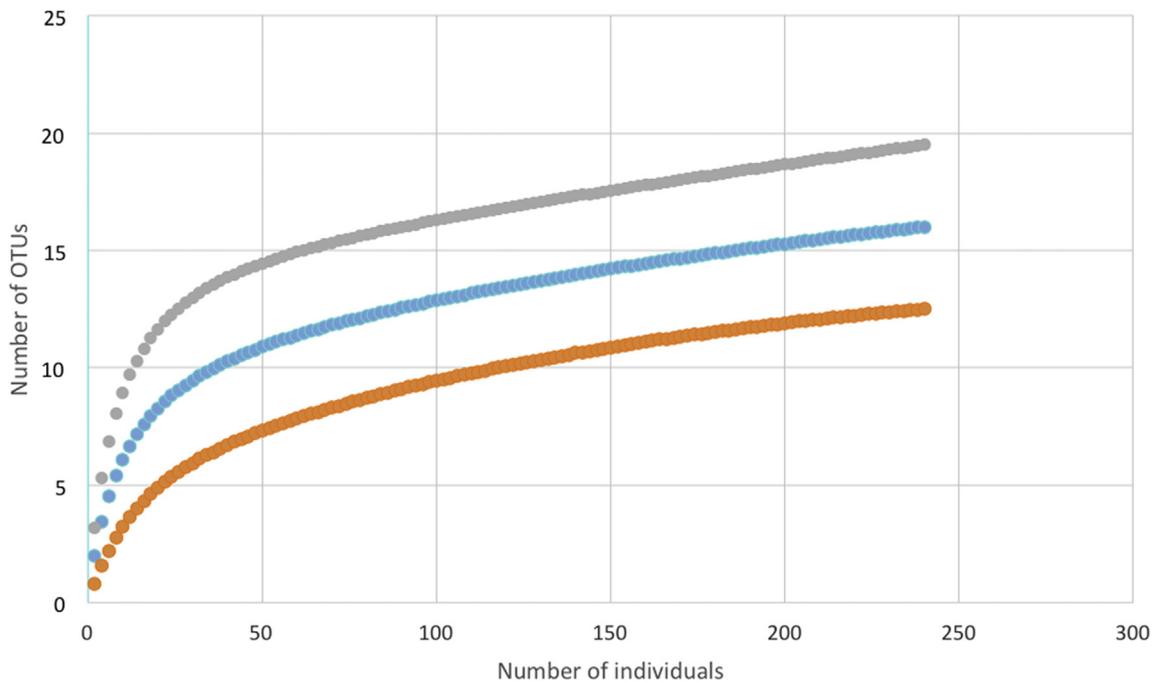
Fig 2 − Species accumulation curve showing the relation between the number of individuals (plants sampled) tested and the total number of taxa obtained. Middle line: number of fungal taxa calculated by the Mao Tau algorithm. Upper and lower lines: 95 % confidence limits of the estimate of taxa number. The curve is based on 100 randomizations.

vineyard, all of them under conventional treatment, with the exception of *Athelia* sp., suggesting a higher diversity of fungal endophytes species in conventional vineyards.

From the 16 fungal OTU identified, four were detected solely in conventional vineyards (*Alternaria porri*, *A. solani*, *Penicillium* sp., and *P. brevicompactum*), two only in biological vineyards (*Athelia* sp. and *D. seriata*) and ten in both modes (Fig 3A).

Most common isolates (over 60 %) in conventional vineyards belonged to *A. porri*, *A. solani*, *Botrytis cinerea*, *Epicoccum nigrum*, *Epicoccum* sp., *P. brevicompactum*, and *Penicillium* sp.; and in biological vineyards, they belonged to *Alternaria alternata*, *Athelia* sp., *Cladosporium* sp., *D. seriata*, and *Nigrospora oryzae*. *Alternaria tenuissima*, *Alternaria* sp., *Colletotrichum* sp., and *Gloetinia* sp. isolates were equally common in conventional and biological vineyards.

From the 16 fungal OTU identified, 15 were detected in Syrah cultivars, 11 in Cabernet Sauvignon and nine in Aragonez. Five OTUs were detected only in Syrah cultivars (*A. solani*, *A. tenuissima*, *Athelia* sp., *D. seriata* and *Penicillium* sp.), one was detected only in Cabernet Sauvignon (*P. brevicompactum*) and none was solely detected in Aragonez cultivar. Nine OTUs were detected in all three cultivars and *A. porri* was detected in Syrah and Cabernet cultivars (Fig 3B).

As for the total number of isolates, 39 % were detected in cv. Syrah vines, 33 % in cv. Cabernet Sauvignon and 28 % in cv. Aragonez. OTUs that most contributed to cv. Syrah values were *A. solani*, *A. tenuissima*, *Athelia* sp., *B. cinerea*, *Colletotrichum* sp., *D. seriata*, *Epicoccum* sp., *N. oryzae*, and *Penicillium* sp. *Alternaria* sp., *E. nigrum*, *Gloetinia* sp., and *P. brevicompactum* were dominant in cv. Cabernet Sauvignon vines. *A. alternata* was the only OTU with more isolates detected in Aragonez than in any other cultivar.

Number and distribution of the isolates of the different OTU was similar in the two localities sampled (data not shown).

The Simpson and Shannon diversity indexes indicate that fungal diversity and evenness did not vary significantly between grapevines under different management modes. As for the cultivars, Syrah presented the highest diversity index values (Table 2).

Multivariate data analysis

The total number of fungi detected was significantly ($p < 0.02$) higher on the conventional than on the biological management (mean number of fungi $\pm$ SE of 2.17 $\pm$ 0.12 *versus* 1.83 $\pm$ 0.10). This was verified for the three cultivars. In biological management, the mean number of fungi $\pm$ SE was 2.10 $\pm$ 0.19 in cultivar Syrah, 1.85 $\pm$ 0.19 in Cabernet Sauvignon and 1.55 $\pm$ 0.16 in Aragonez. In conventional management, the mean number of fungi $\pm$ SE was 2.55 $\pm$ 0.23 in cultivar Syrah, 2.05 $\pm$ 0.21 in Cabernet Sauvignon and 1.90 $\pm$ 0.20 in Aragonez (Fig 4).

The PCA ordination of the fungal species showed that the first two components (PC1, 19.8 % and PC2, 17.6 %) accounted for 37.4 % of the variability of the data. PCA ordination separated samples collected under the conventional mode from the samples from the biological management, *Epicoccum nigrum*, *Epicoccum* sp., and *Botrytis cinerea* were shown to be more dominant in conventional mode and showed a marked separation from *Alternaria alternata*, *Colletotrichum* sp., *Nigrospora oryzae*, and *Cladosporium* sp. that are dominant in biological type of management (Fig 5).

PERMANOVA analysis revealed significant differences in the factor 'Cultivar' ($p < 0.0105$). In biological management,

A

B
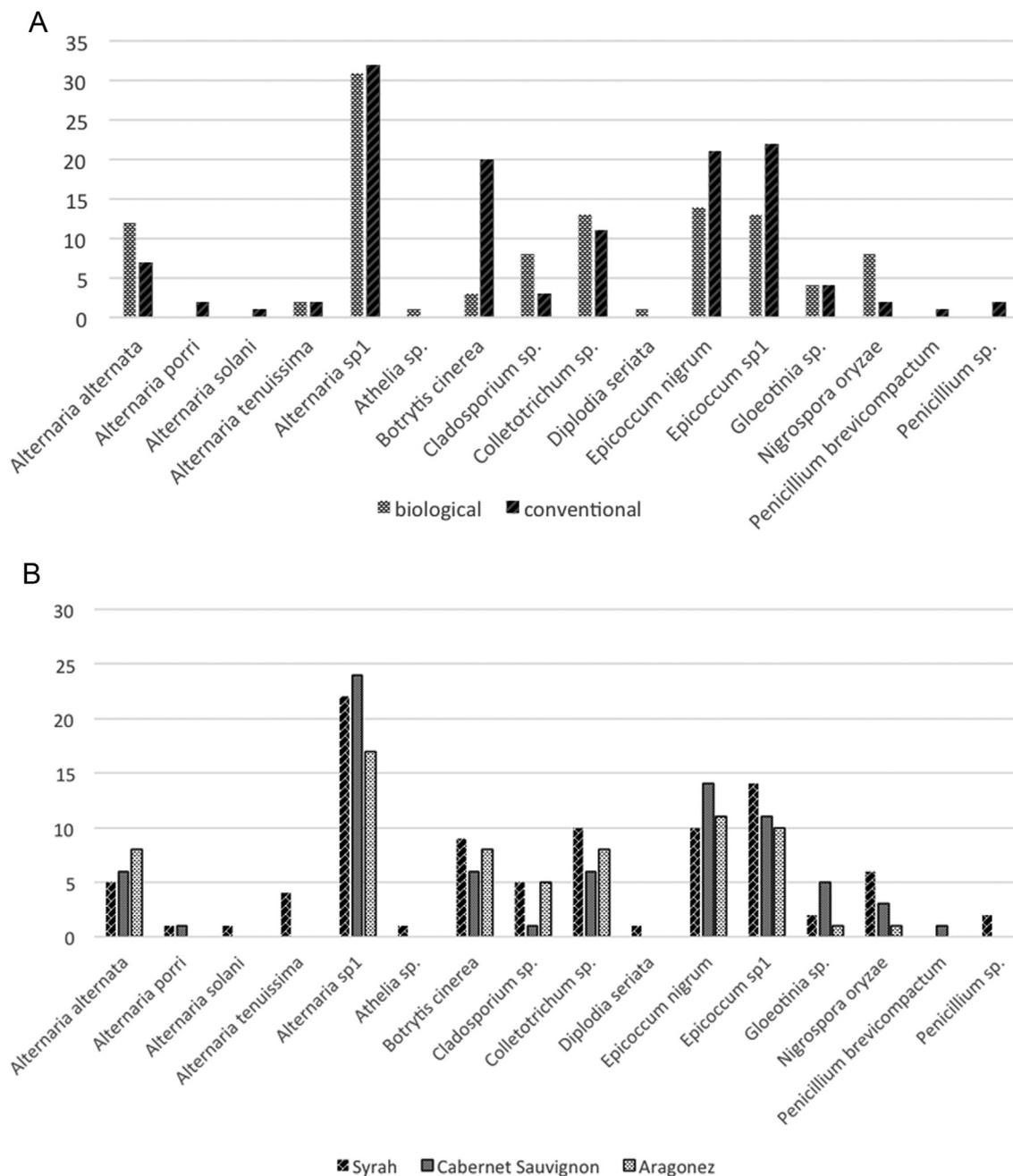
Fig 3 — Number of fungal isolates per OTU identified in the vineyards under study according to type of management (A) and cultivar (B).

Table 2 — Species richness, diversity, and evenness of phyllosphere endophytic fungi from grape obtained in this study.

		Species richness	Simpson diversity	Shannon diversity	Evenness
Mode	Conventional	14	6,6	2,12	0,8
	Biological	12	6,73	2,124	0,85
Cultivar	Aragonez	9	6,53	1,98	0,9
	Cabernet	11	5,86	1,89	0,79
	Syrah	15	8,05	2,32	0,86
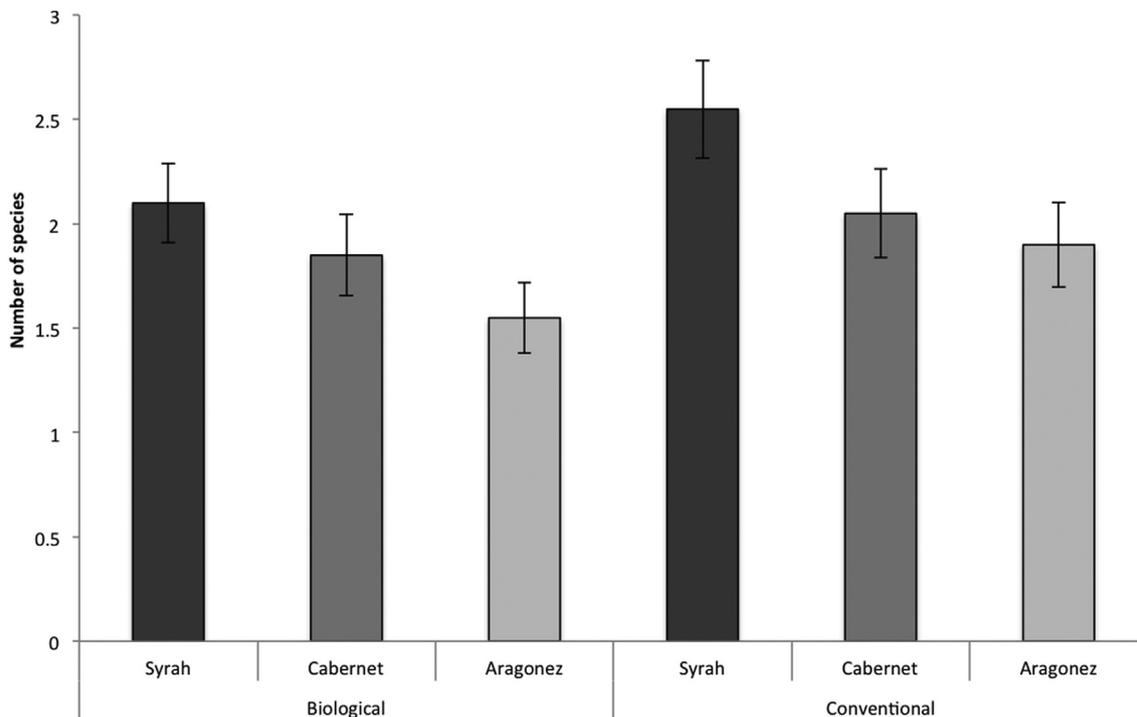
Fig 4 – **Mean number of endophytic fungal OTUs ± standard error (SE) present in the phyllosphere of each cultivar (Syrah, Cabernet Sauvignon and Aragonez) under two different types of management (biological and conventional).**

individual pairwise comparisons detected a significantly higher number of fungi on the Syrah cultivar than Aragonez cultivar (Pairwise Tests, $p_{syrah\ versus\ aragonez} < 0.0399$), however, no significant differences were revealed between Syrah and Cabernet (Pairwise Tests, $p_{syrah\ versus\ cabernet} < 0.3587$), or between Cabernet and Aragonez (Pairwise Tests, $p_{aragonez\ versus\ cabernet} < 0.2367$). In conventional management, individual pairwise comparisons also detected a significantly higher number of fungi on the Syrah cultivar than Aragonez cultivar (Pairwise Tests, $p_{syrah\ versus\ aragonez} < 0.0395$), and no significant differences between Syrah and Cabernet (Pairwise Tests, $p_{syrah\ versus\ cabernet} < 0.1249$), or between Cabernet and Aragonez (Pairwise Tests, $p_{aragonez\ versus\ cabernet} < 0.5955$). In general, the number of fungi was significant consistently higher on Syrah cultivar than Aragonez and Cabernet cultivars (Pairwise Tests, $p_{syrah\ versus\ aragonez} < 0.0032$, $p_{syrah\ versus\ cabernet} < 0.082$, $p_{aragonez\ versus\ aragonez} < 0.2194$).

The PCA ordination of the fungal species in the factor 'Cultivar' showed that the first two components (PC1, 19.8 % and PC2, 17.6 %) accounted for 37.4 % of the variability of the data. PCA ordination did not separate samples according to the cultivar (Fig 6).

Discussion

This study describes the composition of endophytic fungal communities within the plant phyllosphere of three cultivars of grapevine from Alentejo, under different modes of management. Endophytes were isolated in fall, at the end of the vegetative cycle, before yellowing and falling of leaves. Leaves present the highest frequency and diversity of fungal endophytes (Gonzalez & Tello 2011) due to the less barriers fungi face for infection when compared to other parts of the plant (Arnold & Lutzoni 2007) or to the successful colonization of above-ground fungal endophytes that travel among hosts as spores.

In this study, the cumulative curves for species richness approached asymptotic growth (Fig 2), suggesting that most fungal species were detected and sample was representative. Similar accumulation curves were obtained for each cultivar and each management mode analysed individually (data not shown). The species abundance of fungal endophytes in grapevine was mostly comprised of frequent taxa (75 %) rather than rare species (singletons), meaning that there is a large proportion of species that were repeatedly isolated and that showed to be characteristic in grapevine, regardless of the mode of management or cultivar. This result is in agreement with other studies performed in grapevine adult trees (Casieri et al. 2009; Gonzalez & Tello 2011; Pancher et al. 2012) and may be due to the fact that some endophyte OTUs become specialized on plant tissues and occupy a specific ecological role in the plant. The stable and strong colonization of these fungi may then prevent the colonization of new different species.

We achieved ITS PCR amplification for all 240 fungal isolates obtained and sequence analyses placed them into 16 different OTUs, 15 of which belonged to Ascomycota and one to Basidiomycota. Although some authors suggest that basidiomycetes constitute an important part of endophytic communities and low proportions of basidiomycetes may just
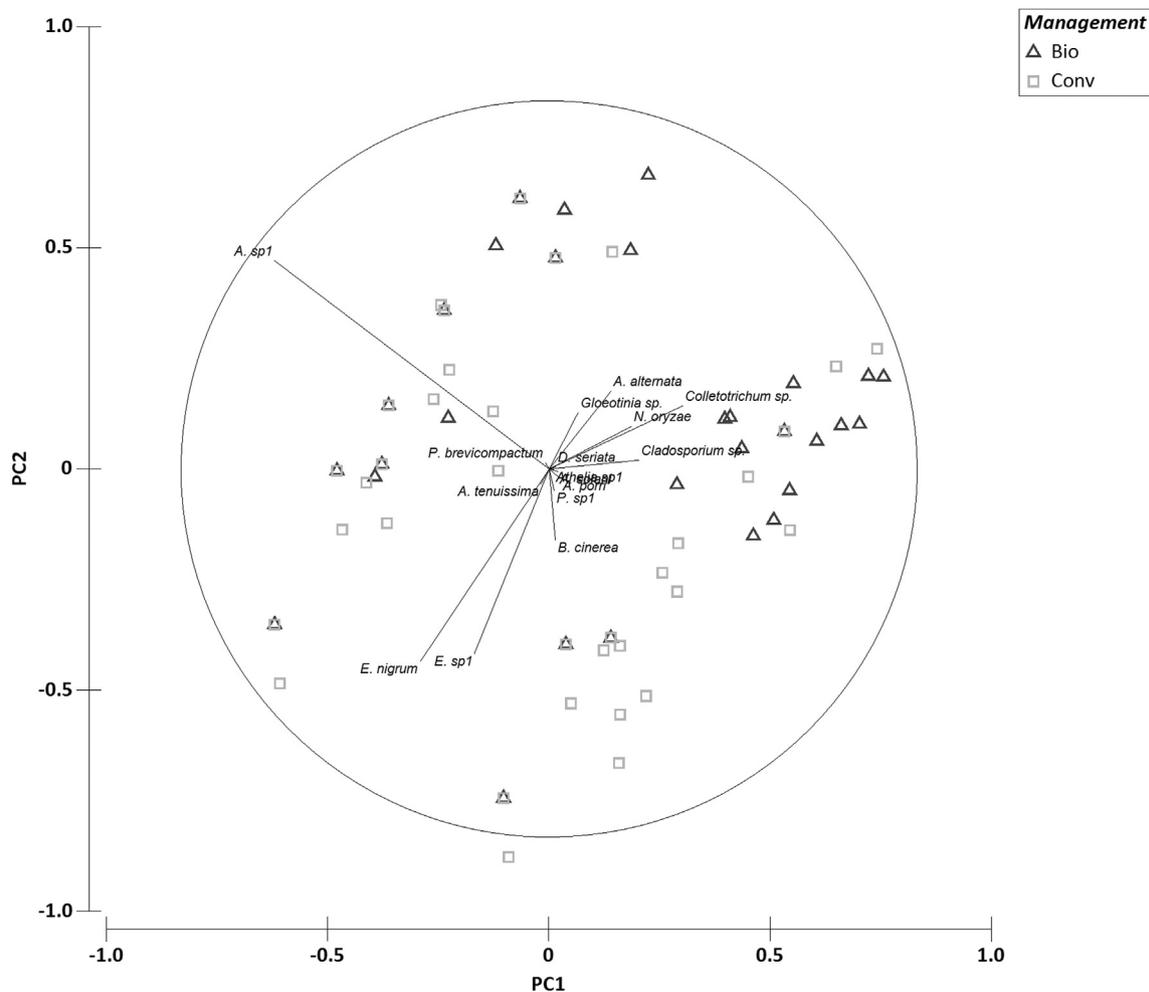
Fig 5 – PCA plot based on presence and absence of fungal OTUs detected as phyllosphere endophytes under two different types of management 'Biological' and 'Conventional' (PC1, 19.8 % and PC2, 17.6 %).

sometimes reflect sampling bias (Mueller *et al.* 2004; Pinruan *et al.* 2010), the predominance of ascomycetes fungi over the basidiomycetes found in this study is consistent with other endophytic studies concerning grapevine (Mostert *et al.* 2000; Gonzalez & Tello 2011; Pancher *et al.* 2012) and other woody plants (Arnold 2007). Within Ascomycota, the Dothideomycetes were the most representative (71 % of total isolates) and within those, the Pleosporales (66 %), which have shown to be one of the main components of the endophytic mycota of many woody plants including grapevine (Gonzalez & Tello 2011; Pancher *et al.* 2012). This is mainly due to *Alternaria* and *Epicoccum* species that are the most frequent among fungal endophytes in grapevine, as well as in other plants (Gonzalez & Tello 2011; Grisan *et al.* 2011; Pancher *et al.* 2012; Landum *et al.* 2016) and have been studied as promising biocontrol agents. *Alternaria* species are usually the principal fungal component of endophytic communities in phyllospheres, mostly due to their particular life style, producing highly melanised hyphae capable to resist and grow under intense UV radiations. *Alternaria* species (37 % of total isolates; 50 % in biological and 50 % in conventional), have shown antagonistic effects against *Botrytis cinerea* and grapevine downy

mildew caused by *Plasmopara viticola* (Dugan *et al.* 2002; Musetti *et al.* 2006). The genus *Epicoccum* comprises 29 % of total isolates obtained in this study (average > 61 % in conventional mode). This classical endophytic genus has also been reported to possess several antifungal properties against grapevine pathogens such as *P. viticola* or *B. cinerea* (Fowler *et al.* 1999). *Epicoccum nigrum* is being developed commercially due to its capability to produce secondary metabolites with antibiotic activity (Martini *et al.* 2009). In summary, 66 % of the isolates obtained in this study belonged either to *Alternaria* or to *Epicoccum* species, which means that endophytic communities may constitute a source of biocontrol agents useful to control important vine diseases. Although *Alternaria* sp. was found in similar levels in both modes, *Epicoccum* species were dominant in the conventional mode.

In general, fungi isolated in this study have been previously reported as grapevine endophytes (Gonzalez & Tello 2011; Pancher *et al.* 2012). A low incidence of pathogenic species was detected. With the exception of *B. cinerea* and *Diplodia seriata*, no pathogenic species, such as esca, Petri disease and excoriose related fungi were detected, in contrast to other studies that frequently report high isolation rates of *P. viticola*
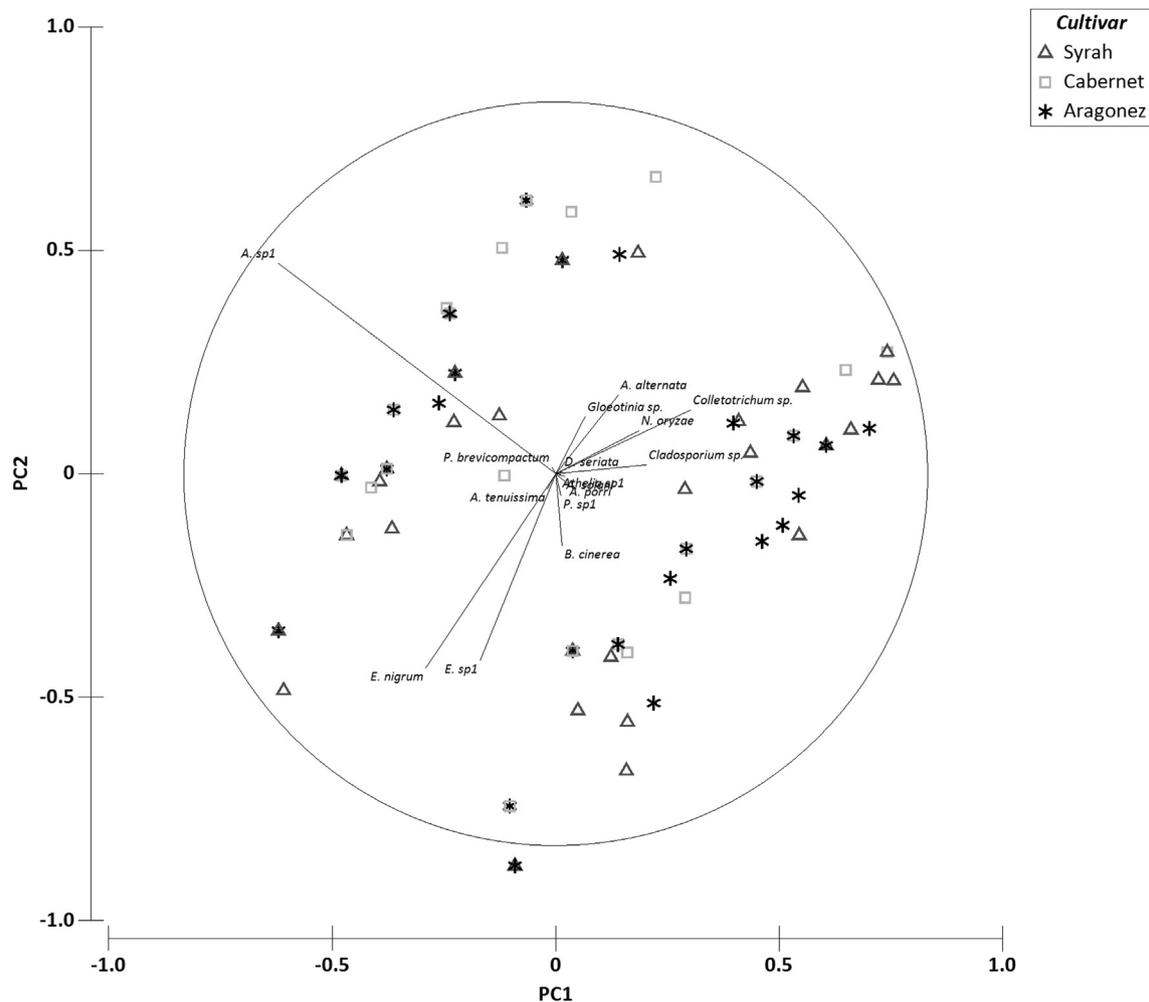
Fig 6 − **PCA plot based on presence and absence of fungal OTUs detected as phyllosphere endophytes of three different grape cultivars 'Syrah', 'Aragonez', and 'Cabernet Sauvignon' (PC1, 19.8 % and PC2, 17.6 %).**

(Rodolfi *et al.* 2006; Gonzalez & Tello 2011). The common occurrence of *B. cinerea* species as a grapevine endophyte has been frequently reported (Casieri *et al.* 2009; Gonzalez & Tello 2011), suggesting that this pathogen is latent, behaving as a plant endophyte, but may become pathogenic under specific physiological or environmental conditions. In addition, *Aureobasidium pullulans* was not isolated from grapevines in this study. This fungus has shown to be very frequent in grapevine plants (Martini *et al.* 2009; Gonzalez & Tello 2011; Grisan *et al.* 2011; Schmid *et al.* 2011; Pancher *et al.* 2012) and its role as antagonist against several pathogens has led to its commercial development to control *B. cinerea*. Differences found may be due to the cultivars and environmental dissimilarities, which have shown to have a very important impact in the fungi present (Núñez-Trujillo *et al.* 2012; Pancher *et al.* 2012).

A higher number of fungi, as well as of OTUs, was observed in grapevines from Syrah cultivar and the lower number of fungi and OTUs was observed in Aragonez. These differences were significant despite the mode of management. Differences in number of fungi were only significant between Syrah and Aragonez. Differences in fungal communities of different

grapevine cultivars have been reported (Casieri *et al.* 2009; Gonzalez & Tello 2011), but some studies showed no influence in fungal endophyte composition when comparing cultivars (Pancher *et al.* 2012). Differences in fungal composition of the different cultivars may be related to different plant breeding and selection processes which cultivars have been exposed to; different sugar content, pH and nutrient composition; differences in phenological stages; or presence and abundance of secondary metabolites produced by the different cultivars. Among the three cultivars used here, some characteristics may partially explain the similarities and differences in fungal endophytic communities. Aragonez is the most different; it has its origin in the Iberian Peninsula and has a higher genetic variability when comparing to Syrah and Cabernet. The latter are among the most used cultivars in the world, they are original from France and very similar in terms of maturation and aroma intensity. Some OTUs were observed only in one of the cultivars, however the low number of isolates (<4) these OTUs present, is not enough to establish a relation with the cultivar, as shown in the PCA (Fig 6), where a homogeneous spread of cultivars is observed. In addition, as for OTUs with more than eight isolates, the

percentage of isolates in each cultivar never exceeded 60 % and maybe for that reason, again no relation could be established between cultivar and OTU.

When comparing grapevines under the different modes of management, it was observed that the total number of fungi detected was significantly ($p < 0.02$) higher on the conventional than on the biological mode (54 % *versus* 46 %) which was not expected as conventional treatments have impact on non-target organisms and biological practices are usually linked to higher microbial populations and community diversities (Araújo *et al.* 2009). This means that products used in biological modes, such as copper, may also have impacts on the microbial communities and should not be disregarded. The higher number of OTUs obtained in the conventional mode than in biological mode did not, however, result in higher diversity indexes, much due to the also higher number of isolates obtained in the conventional mode. Simpson diversity and Shannon diversity indexes show very similar values in both modes (Table 2), with a slight higher diversity value in the biological mode, as observed in previous studies (Pancher *et al.* 2012). Fungal endophyte composition also showed some differences between both modes. *Athelia* sp. and *D. seriata* were only detected in the biological mode and *Alternaria porri*, *Alternaria solani*, *Penicillium* sp., and *Penicillium brevicompactum* were only detected in the conventional mode. In addition, *B. cinerea*, *Epicoccum* sp., and *E. nigrum* showed to be more frequent in conventional vineyards (87 %, 63 %, and 60 %, respectively) and *Nigrospora oryzae*, *Cladosporium* sp., and *Alternaria alternata* were more frequent in biological vineyards (80 %, 73 %, and 63 %, respectively). This shows that composition of phyllosphere fungal communities is different upon the type of culture management. PCA analysis reinforced that fungal community composition showed a separation between the biological and the conventional vineyards (Fig 5). Summing up, plants under the two modes of management revealed differences in terms of total fungal endophyte number, fungal diversity and fungal communities composition. These differences may be related to the use of chemical/organic products that directly affect microorganisms, or to alterations in plant physiology and consequently on plant associated microorganisms. The response of plant associated microbial communities to external products is of great interest for agriculture and further work should focus on the response of plant endophytes to such substances. In addition, some studies have shown that management modes may also interfere on endophytes role, as higher antagonistic effect was observed in endophytes isolated from plants under organic management (Schmid *et al.* 2011; Pancher *et al.* 2012). Further studies are needed to test this hypothesis, but this would mean that differences between the two modes of management could be even greater. It would also be interesting to test if antagonistic activities increase due to interactions/synergistic effects of the different fungal species present in the endophytic community. In addition, antagonistic capabilities in the same endophytic fungal species also may vary between plant species, age of the host or plant tissue sampled (Saikkonen *et al.* 1998). One of the future challenges will be to identify functional differences among endophytic fungi under different conditions.

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