Diversity of *Colletotrichum* Species Associated with Olive Anthracnose and New Perspectives on Controlling the Disease in Portugal

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Abstract: Olive anthracnose is a very common and severe disease caused by diverse species of fungi belonging to *Colletotrichum acutatum* and *Colletotrichum gloeosporioides* complexes. To understand aspects of the *Colletotrichum* colonization and primary infection in olives, *Colletotrichum* spp. were isolated from the interior of 2-year stems, flower buds, and immature fruits of three important olive cultivars, Galega vulgar, Cobrançosa, and Azeiteira, from different sites within Alentejo, a major olive-producing region in Portugal. A total of 270 trees was sampled, and 68 *Colletotrichum* spp. isolates were obtained from 46 olive trees. DNA extraction and amplification of *β-tubulin* and *GADPH* genes through PCR revealed that the vast majority of the isolates showed high similarity to *Colletotrichum nymphaeae*, and only three isolates showed high similarity to *Colletotrichum godetiae*. The highest number of *Colletotrichum* spp. isolates was detected in olive trees from Galega vulgar and in immature fruits. No significant differences in the number of *Colletotrichum* spp. isolates were found in trees from different sites. The highest percentages of infected immature fruits were obtained in trees that also presented a high percentage of 2-year stem infections, which may indicate that 2-year stems serve as important sources of inoculum, and the fungus may travel from the stems to other parts of the plant. Another indication of such possibility is that one isolate of *C. nymphaeae* (*C. nymphaeae* 2), characterized by a unique nucleotide mutation within the beta tubulin gene, was present in different organs of the same tree, both in 2-year stems and in recently formed vegetative organs as flower buds and immature fruits, which seem to suggest that it may be the same isolate, which has moved systemically inside the plant. The results presented here can play an important role in working out strategies for the effective and timely management of the disease and in reducing the number of unnecessary fungicide applications.

Keywords: anthracnose; olive cultivars; control; plant organs

1. Introduction

The olive tree (*Olea europaea* L.) is affected by several diseases including anthracnose, a major concern in most olive-producing countries, which is able to destroy the entire production [1–4]. Anthracnose is caused by diverse species of fungi belonging to genus *Colletotrichum* [5,6]. Some *Colletotrichum* species, previously classified as *Colletotrichum acutatum* and then included within the *C. acutatum* complex (*Colletotrichum nymphaeae, Colletotrichum fiorinae, Colletotrichum godetiae, C. acutatum, Colletotrichum rhombiforme,* and *Colletotrichum simmondsii*), highly prevail in areas where the disease occurs epidemically [3]. In Portugal, the species *C. nymphaeae, C. acutatum*, and *C. godetiae* together reach levels of over 95% [6].

The disease typically affects fruits near maturation and, consequently, the quality of the fruits and oil obtained (high acidity, off-flavor, reddish color, and a considerable reduction of polyphenols, α-tocopherol, and β-sitosterol) [7]. Under moist conditions, infected fruits develop dark, necrotic, circular, sunken lesions with an abundant production of orange-colored masses of spores on the surface, leading to premature fruit drop, as well as fruit rot; in dry weather, mummification occurs, frequently leading to total yield losses [2,4,8]. The pathogen can also be present on flowers, leaves, shoots, and branches and may cause blossom blight, chlorosis, and necrosis of the leaves in the early spring and severe defoliation and wilting in the late spring and early summer, as well as dieback of the branches, with the latter being associated with toxins produced by the pathogen [1,9,10].

In addition to environmental conditions (e.g., humidity, rain, and temperature), the virulence of the pathogen, the maturity and integrity of the fruits, and the olive cultivar have also been associated with the disease incidence [3,4]. In Portugal, the main olive oil cultivar, Galega vulgar, is known to be very susceptible to anthracnose, Cobrançosa is moderately tolerant, and Azeiteira is considered to be resistant [10,11].

Conidia from *Colletotrichum* spp. germinate from acervuli on tree mummified fruits, leaves, and twigs and are dispersed through rain during the fall when the fruits begin to ripen, becoming the primary inoculum of the disease [1,9,12,13]. The pathogen sporulates on the surface of rotten fruits, and the spores give rise to secondary infection cycles. Moral et al. [14] showed that in the spring, leaves, shoots, flowers, and young fruits become infected, but the infection remains latent and may be an important source of inoculum for autumn epidemics [12,14,15]. The complete disease cycle of olive anthracnose is still not fully understood [9]. It is not known if the pathogen can travel from latent infected branches or other infected organs to other organs, such as flowers and fruits, which were not directly infected. If that happens, once a plant becomes infected, the plant may act as a reservoir and will not be dependent on a new infection to initiate the disease. If so, fungicide treatments before flowering would protect flowers from early infections but would not be efficient for already existing latent infections. At present, the control of the disease is based on the application of copper products or penetrating products, such as trifloxystrobin, during autumn, and no systemic products are used. In this context, the study of internal infections and their possible circulation on vascular organs would be extremely useful for the improvement of management choices, namely the timing and the use of systemic fungicides.

Building on the latter knowledge, the aim of the present study was to understand aspects of *Colletotrichum* colonization and the primary infection of olive anthracnose. For that, the presence of *Colletotrichum* spp. was evaluated in the interior of different organs from three major Portuguese olive cultivars with different degrees of susceptibility to olive anthracnose, grown in different sites. The following hypotheses were tested: (i) there would be significant differences in the presence of *Colletotrichum* spp. in different plant organs; (ii) there would be significant differences in the presence of *Colletotrichum* spp. in different cultivars; and (iii) there would be significant differences in the presence of *Colletotrichum* spp. in different sites.

2. Materials and Methods

2.1. Study Area and Sample Collection

The sampling was carried out during 2016 in three important olive oil-producing sites within the Alentejo region (southern Portugal), all influenced by the Mediterranean climate: In Vidigueira (38°10′01.17″ N, 7°44′16.75″ W), the altitude is 156 m above sea level, the mean temperature is 15.0 °C, the annual rainfall is approximately 600 mm, and the soils are of granite origin. In Monforte (39°4′3.99″ N, 7°28′13″ W), the altitude is 376 m above sea level, the mean temperature is 16.5 °C, the annual rainfall is approximately 660 mm, and the soils are mostly of schist and calcareous origin. In Elvas (38°54′31.34″ N, 7°8′43.52″ W), the altitude is 220 m above sea level, the mean temperature is 16.3 °C, the annual rainfall is approximately 598 mm, and the soils are mostly of schist and calcareous origin. All the olive trees sampled were of medium size (with ages ranging from 10 to 30 years) and were planted at a spacing of 7 × 5 m. The sampled olive groves occupied an area of 320,000 m^2 in Monforte, 150,000 m^2 in Vidigueira, and 30,000 m^2 in Elvas, and all were produced under an intensive regime. All the experimental olive groves included programmed applications of fungicide and insecticide products, such as copper hydroxide, trifloxystrobin, deltamethrin, and dimethoate.

The sampled olive trees belonged to three different cultivars (Galega vulgar, Cobrançosa, and Azeiteira) and did not present any visible anthracnose symptoms. At each site, the area of olive trees from each cultivar was divided into 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. A total of 270 trees was sampled (3 sites × 3 cultivars × 30 trees per cultivar). The sampling was repeated at 3 different periods, for each type of plant organ; 2-year stems (early spring), flower buds (late spring), and immature fruits (early fall), totaling 810 samples (270 trees × 3 periods). For each tree and for each plant organ, 50 samples were collected around the whole tree at 1.5 m above the ground. The sampling was always made before the applications of the chemical products. The samples were transported to the laboratory in a refrigerated basket, stored at 4 °C, and processed within the next 48 h.

2.2. Isolation of Colletotrichum spp.

To suppress the epiphytic micro-organisms on the field-collected samples, 2-year stems, flower buds, and immature fruits were surface disinfected. The disinfection involved a sequence of 3-min immersions in 96% ethanol, 3% sodium hypochlorite solution, 70% ethanol, and 3 times in ultra-pure water, respectively, and then, the samples were dried in sterile Whatman paper. The 2-year stems were cut into 0.5 cm^2 sections; the flower buds and immature fruits were cut lengthwise and placed (6 pieces per plate) on 9-cm diameter Petri dishes containing potato dextrose agar medium (PDA) (Merck, Darmstadt, Germany). The entire procedure was performed inside a sterile laminar airflow chamber. The plates were subsequently incubated in darkness at 23–25 °C for 4 days.

Colletotrichum spp. were selected by morphological characteristics, such as the rate of growth, mycelium color, texture, nature of the growing margin, and color of the reverse side. The shape of the conidia was observed under an Olympus BX-50 compound microscope (1000× magnification). The fungus was then isolated by transferring a colony to a new (PDA) plate for growing. Mycelia from isolated *Colletotrichum* spp. were ground in liquid nitrogen and used in DNA extraction for further identification of the species.

2.3. Fungal DNA Extraction and Identification

The DNA extraction was performed using the Cetyltrimethylammonium ammonium bromide (CTAB) method [16], with some modifications as described previously [17]. The DNA concentration was determined by using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA).

The fungal isolates were identified by PCR amplification of the internal transcribed spacer (*ITS*) region (ITS1, 5.8S rRNA, ITS2), part of the *β-tubulin 2* (*tub2*) gene, and the intron of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) by using primers ITS1 and ITS4 [18], T1 and T22 [19], and GDFfwd and GDFrev [20,21], respectively. The PCR reactions were performed in a 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 MgCl2, 0.2 mM dNTPs (Fermentas, Thermo Scientific, Waltham, MA, USA), 0.2 µM of each primer, and 2.5 U of DreamTaq DNA polymerase (Fermentas). The amplification reactions were carried out in a Thermal Cycler (BioRad, Hercules, CA, USA) with an initial temperature of 95 °C for 2 min, followed by 40 cycles of 95 °C for 30 s; 55 °C for 45 s (for *ITS*), 58 °C for 55 s (for *tub2*), and 56 °C for 55 s (for *GAPDH*); and 72 °C for 60 s, as well as a final extension at 72 °C for 10 min.

The amplified products were analyzed by agarose gel electrophoresis. The PCR products were purified using DNA Clean & Concentrator (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions and sequenced in both directions by Macrogen (Madrid, Spain). The search for homologous sequences was done using basic local alignment search tools (BLAST) at the National Center for Biotechnology Information (NCBI). The analysis of the *ITS*, *tub2*, and *GAPDH* sequences was carried out using CLUSTAL W in MEGA software version 7.0 [22]. The phylogenetic relationships were inferred using neighbor joining (NJ), and the trees were produced using the minimum evolution, maximum parsimony, and maximum likelihood methods in the MEGA 7 software. The bootstrap analyses with 1000 replicates were performed to evaluate the significance of the interior branches.

2.4. Multivariate Data Analysis

Multivariate analyses were performed to detect significant differences in the total number of olive trees showing the presence of *Colletotrichum* spp. in three different plant organs "2-year stems, flower buds, and immature fruits"; in three cultivars, "Galega vulgar, Cobrançosa, and Azeiteira", and in three sites "Vidigueira, Monforte, and Elvas". The statistical analyses of the data were performed using the PRIMER v6 software package [23] with the PERMANOVA add-on package [24]. The total number of olive trees with the presence of *Colletotrichum* spp. was calculated using the dataset from different plant organs, in each cultivar, and at each site. A three-way permutational analysis of variance (PERMANOVA) was applied to test the hypothesis that significant differences existed in the total number of trees with *Colletotrichum* spp. among "2-year stems, flower buds, and immature fruits", among "Galega vulgar, Cobrançosa, and Azeiteira", and among "Vidigueira, Monforte, and Elvas". The PERMANOVA analysis was carried out following the two-factor design: organs: "2-year stems, flower buds, and immature fruits" (3 levels, fixed); cultivars: "Galega vulgar, Cobrançosa, and Azeiteira" (3 levels, random); and sites: "Vidigueira, Monforte, and Elvas" (3 levels, random, nested in cultivars). The total data were square root transformed in order to scale down the importance of highly abundant replicates and increase the importance of the less abundant ones in the analysis of similarity. The PERMANOVA analysis was conducted on a Bray–Curtis similarity matrix [25]. 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 the presence of *Colletotrichum* spp., was performed to explore patterns in the multidimensional data by reducing the number of dimensions with minimal loss of information. The PCA ordination was based on each of the three sites "Vidigueira", "Monforte", and "Elvas" and on each of the three cultivars "Galega vulgar", "Cobrançosa", and "Azeiteira". Prior to the calculation of the PCA, the ordination data were checked for normal distribution and, if necessary, were log (X + 1) transformed prior to analysis, and then, the data were normalized by subtracting the mean and dividing by the standard deviation for each variable.

The relative contribution of the presence of *Colletotrichum* spp. in 2-year stems, flower buds, and immature fruits to the average of similarity and dissimilarity between the a priori defined groups; sites (Monforte, Vidigueira, and Elvas); and cultivars (Galega, Cobrançosa, and Azeiteira) was calculated using the two-way crossed similarity percentage analysis (SIMPER, cut-off percentage: 100%).

3. Results

3.1. Isolation, Amplification, and Identification of Colletotrichum spp.

Identical isolates within the same tree of the same plant organ (2-year stems, flower buds, and immature fruits) were considered to be a unique isolate. A total of 68 isolates with morphological and cultural characteristics of *Colletotrichum* spp. was obtained from the 810 olive samples (270 trees × 3 sampling periods). In the 68 isolates, the conidia of a fusiform shape were observed microscopically, and colonies varied from slow to fast growing and from pink to orange colors. The analysis of the *ITS* rDNA of all the isolates resulted in highly similar sequences corresponding to five different *Colletotrichum* species, and the analysis was unable to discriminate amongst them. The identification of *Colletotrichum* at the species level was achieved through the analysis of *tub2* (1500-bp) and *GAPDH* (200-bp) partial genes. The sequence alignment and phylogenetic analysis of the *tub2* and *GAPDH* sequences allowed the identification of all 68 isolates as belonging to *C. acutatum* complex (*C. nymphaeae* and *C. godetiae)*. The vast majority of the isolates (65) showed a high similarity to *C. nymphaeae* in both *tub2* (Accession number JQ949852.1) and *GAPDH* (Accession number JQ948531.1) sequences from the database, obtained from *Olea europaea* L. in Portugal (Table 1). From those 65 isolates, 45 (69.2%) (*C. nymphaeae* 1) matched 100% with *C. nymphaeae* in both *tub2* and *GAPDH* sequences from the database. Additionally, 8 (12.3%) isolates (*C. nymphaeae* 2) revealed a difference in one nucleotide of the beta tubulin sequence, leading to a synonymous change in amino acid 151 of the *β-tubulin* gene. The other 12 (18.5%) isolates (*C. nymphaeae* 3) presented a difference in one nucleotide of the beta tubulin sequence, leading to a non-synonymous change in amino acid 268 of the *β-tubulin* gene, from a basic and positively charged residue (Arginine) to a non-polar hydrophobic residue (Proline). Three isolates matched with *C. godetiae* in both *tub2* (Accession number JQ950066.1 and JQ950064.1) and *GAPDH* (Accession number JQ948746.1 and JQ948744.1) sequences from the database, obtained from *Olea europaea* L. in Greece and Italy, respectively.

The phylogenetic tree deduced from both the *tub2* and *GAPDH* nucleotide sequence alignments (Figure 1) revealed the segregation of the isolates into five main clusters. As expected from the analysis of sequences, the isolates were grouped according to *Colletotrichum* species. The *C. nymphaeae* cluster (cluster I) appeared to be divided into two subgroups: one with *C. nymphaeae* 1 and 3 together with the Portuguese isolate from the database and the other subgroup with *C. nymphaeae* 2. One other cluster (cluster II) included sequences from isolates of *C. acutatum* obtained from *Olea europaea* L. in Portugal and South Africa (Accession numbers, *tub2*: JQ950014.1 and JQ950015.1 and *GAPDH*: JQ948694.1 and JQ948695.1), respectively. *Colletotrichum fioriniae* isolates obtained in the database sampled in *Olea europaea* L. from Portugal (Accession numbers, *tub2*: JQ949993 and *GAPDH*: JQ948672) formed another cluster (Cluster III). Cluster IV appeared to be divided into three subgroups: one with the *C. godetiae* isolates from this study; another with the isolates obtained in the database from *Olea europaea* L. from Greece (Accession numbers, *tub2*: JQ950066.1 and *GAPDH*: JQ948746) and from Italy (Accession numbers, *tub2*: JQ950065.1 and GAPDH: JQ948745); and another with *C. rhombiforme* isolates from the database from *Olea europaea* L. in Portugal (Accession numbers *tub2*: JQ950108 and *GAPDH*: JQ948788). *Colletotrichum fruticola* isolates obtained in the database from *Fragaria* sp. in the United States of America (Accession numbers, *tub2*: JX010394 and *GAPDH*: JX010035), *Colletotrichum kahawae* isolates obtained in the database from *Olea europaea* L. in Australia (Accession numbers, *tub2*: JX010434 and *GAPDH*: JX009966), and a *Colletotrichum theobromicola* isolate belonging to *Colletotrichum gloeosporioides* species complex from *Olea europaea* L. in Australia (Accession numbers, *tub2*: JX010376.1 and *GAPDH*: JX009953.1) appear in a separate cluster (cluster V) and were used as an outgroup.
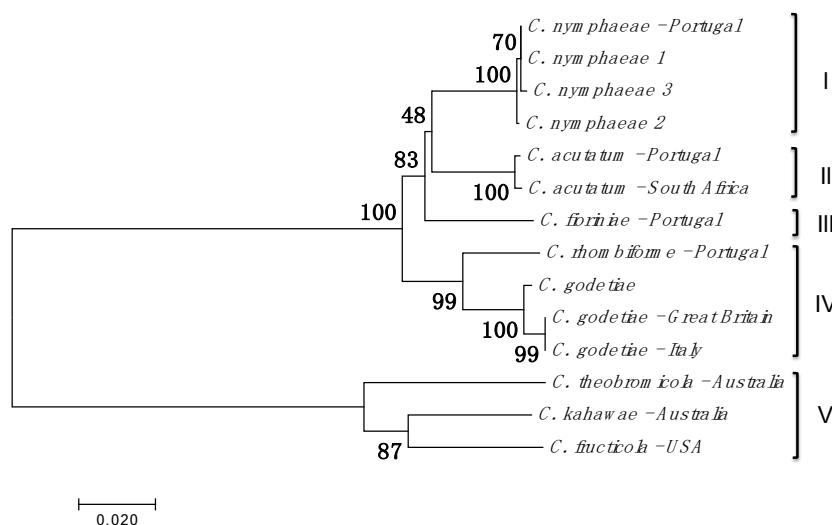
Figure 1. Phylogenetic tree analysis of isolates belonging to the *Colletotrichum acutatum* and *Colletotrichum gloeosporioides* complexes were based on *tub2* and *GAPDH* sequences. The maximum likelihood method, based on the Kimura 2-parameter model, was constructed from the sequence alignment of *C. nymphaeae* 1, *C. nymphaeae* 2, *C. nymphaeae* 3, and *Colletotrichum godetiae* isolates and from sequences retrieved from the GenBank database. Repeated sequences within each isolate were omitted. The phylogenetic analysis included 10 sequences. Multiple sequence alignments were generated using MEGA 7, and the phylogenetic tree was constructed by the neighbor joining (BioNJ algorithms), based on calculations from pairwise nt sequence distances for gene nt analysis. The bootstrap analysis was done with 1000 replicates. The numbers above the lines indicated bootstrap scores out of 1000 replicates.

3.2. Colletotrichum spp. in Olive Cultivars, Sites, and Plant Organs

Colletotrichum spp. were identified in 46 olive trees of the 270 trees sampled (17.0%). Of those 46 trees, 33 (71.7%) belonged to cultivar Galega vulgar, 5 (10.9%) to Cobrançosa, and 8 (17.4%) to Azeiteira and 15 (32.6%) were located in Vidigueira, 13 (28.3%) in Monforte, and 18 (39.1%) in Elvas. In addition, from those 46 olive trees, 8 (15.1%) showed the presence of *Colletotrichum* spp. in the 2-year stems, 12 (22.6%) in the flower buds, and 33 (62.3%) in the immature fruits (Table 1).

Table 1. Presence of each *Colletotrichum* spp. isolate (*C. nymphaeae* 1, *C. nymphaeae* 2, *C. nymphaeae* 3, and *Colletotrichum goditiae*) in the a priori defined groups: sites (Monforte, Vidigueira, and Elvas), cultivars (Galega vulgar, Cobrançosa, and Azeiteira), and plant organs (2-year stems, flower buds, and immature fruits). Each line represents the same olive tree sampled in the three different organs.

Sites	Cultivars	2-Year Stems	Flower Buds	Immature Fruits
Vidigueira	Galega vulgar	-	-	*C. nymphaeae* 1 and 3
		C. nymphaeae 1 and 3	-	-
		-	*C. goditiae*	*C. goditiae*
		C. nymphaeae 1 and 2	-	*C. nymphaeae* 1 and 2
		-	-	*C. nymphaeae* 1
		C. nymphaeae 1	-	*C. nymphaeae* 1
		-	*C. nymphaeae* 1	*C. nymphaeae* 1
		-	-	*C. nymphaeae* 1
		-	-	*C. nymphaeae* 1 and 3
		-	-	*C. nymphaeae* 1
		C. nymphaeae 1	-	-
		C. nymphaeae 1	-	-
		-	-	*C. nymphaeae* 1
	Cobrançosa	-	*C. goditiae*	-
		-	*C. nymphaeae* 1	-

Table 1. *Cont.*

Sites	Cultivars	2-Year Stems	Flower Buds	Immature Fruits
Monforte	Galega vulgar	-	-	*C. nymphaeae* 1
		-	-	*C. nymphaeae* 1
		-	-	*C. nymphaeae* 1
		-	*C. nymphaeae* 1 and 3	*C. nymphaeae* 1
		-	-	*C. nymphaeae* 1
		-	-	*C. nymphaeae* 1
		-	-	*C. nymphaeae* 1
		-	*C. nymphaeae* 1 and 3	-
		-	-	*C. nymphaeae* 1
	Azeiteira	-	*C. nymphaeae* 1	-
		-	-	*C. nymphaeae* 2
		-	*C. nymphaeae* 1	-
		-	-	*C. nymphaeae* 1 and 3
Elvas	Galega vulgar	-	-	*C. nymphaeae* 1 and 3
		-	-	*C. nymphaeae* 1
		C. nymphaeae 1	-	*C. nymphaeae* 1
		-	-	*C. nymphaeae* 1 and 3
		-	-	*C. nymphaeae* 1 and 3
		-	-	*C. nymphaeae* 1
		-	-	*C. nymphaeae* 1
		C. nymphaeae 1	-	-
		-	-	*C. nymphaeae* 1 and 3
		C. nymphaeae 2	*C. nymphaeae* 2	*C. nymphaeae* 2
		-	-	*C. nymphaeae* 1
	Cobrançosa	-	-	*C. nymphaeae* 1 and 3
		-	-	*C. nymphaeae* 1
		-	-	*C. nymphaeae* 2
	Azeiteira	-	*C. nymphaeae* 1	-
		-	*C. nymphaeae* 2	-
		-	-	*C. nymphaeae* 1 and 3
		-	*C. nymphaeae* 1	-
Total isolates (68)		10	14	44

In all the sites, the highest number of trees with *Colletotrichum* spp. was detected in cultivar Galega vulgar: in Vidigueira, 43.3% (vs. 6.7% for Cobrançosa and 0% for Azeiteira); in Monforte, 30.0% (vs. 0% for Cobrançosa and 13.3% for Azeiteira); and in Elvas, 36.7% (vs. 10.0% for Cobrançosa and 13.3% for Azeiteira).

Among all the trees that presented *Colletotrichum* spp., in Vidigueira, the mean percentage in each organ tested was 10.0% in the 2-year stems (ranging from 8% to 12%), 10.0% in the flower buds (10% in two trees), and 24.6% in the immature fruits (ranging from 20% to 32%) in cultivar Galega vulgar; 0% in the 2-year stems and immature fruits and 10.0% in the flower buds (ranging from 8.0% to 12.0%) in cultivar Cobrançosa; and 0% in the 2-year stems, flower buds, and immature fruits sampled in cultivar Azeiteira (Table 2). In Monforte, the mean percentage in each organ tested was 0% in the 2-year stems, 14.0% in the flower buds (ranging from 12.0% to 16.0%), and 48.5% in the immature fruits (ranging from 12.0% to 70.0%) in cultivar Galega vulgar; 0% in the 2-year stems, flower buds, and immature fruits in cultivar Cobrançosa; and 0% in the 2-year stems, 15.0% in the flower buds (ranging from 10.0% to 20.0%), and 28.0% in the immature fruits (ranging from 26.0% to 30.0%) in cultivar Azeiteira (Table 2).

In Elvas, the mean percentage in each organ tested was 16.0% in the 2-year stems (ranging from 14.0% to 18.0%), 16.0% in the flower buds (ranging from 14.0% to 18.0%), and 30.4% in the immature fruits (ranging from 16.0% to 50.0%) in cultivar Galega vulgar; 0% in the 2-year stems and the flower buds and 16.0% in the immature fruits (ranging from 14.0% to 18.0%) in cultivar Cobrançosa; and 0%

in the 2-year stems, 16.0% in the flower buds (ranging from 14% to 18%), and 30.0% in the immature fruits (in only one tree) in cultivar Azeiteira (Table 2).

Table 2. The percentage of infected trees and infected organs in each of the a priori defined groups: sites (Monforte, Vidigueira, and Elvas), cultivars (Galega vulgar, Cobrançosa, and Azeiteira), and plant organs (2-year stems, flower buds, and immature fruits).

Site	Vidigueira 16.7%								
Cultivar	Galega Vulgar			Cobrançosa			Azeiteira		
Infected trees	43.3%			6.7%			0%		
	2-Year Stems	Flower Buds	Immature Fruits	2-Year Stems	Flower Buds	Immature Fruits	2-Year Stems	Flower Buds	Immature Fruits
Infected organ	10.0%	10.0%	24.6%	0%	10.0%	0%	0%	0%	0%
Site	Monforte 14.4%								
Cultivar	Galega Vulgar			Cobrançosa			Azeiteira		
Infected trees	30.0%			0%			13.3%		
	2-Year Stems	Flower Buds	Immature Fruits	2-Year Stems	Flower Buds	Immature Fruits	2-Year Stems	Flower Buds	Immature Fruits
Infected organ	0%	14.0%	48.5%	0%	0%	0%	0%	15.0%	28.0%
Site	Elvas 20.0%								
Cultivar	Galega Vulgar			Cobrançosa			Azeiteira		
Infected trees	36.7%			10.0%			13.3%		
	2-Year Stems	Flower Buds	Immature Fruits	2-Year Stems	Flower Buds	Immature Fruits	2-Year Stems	Flower Buds	Immature Fruits
Infected organ	16.0%	16.0%	30.4%	0%	0%	16.0%	0%	16.0%	30.0%

The vast majority of positive trees (39 out of 46) showed the presence of *Colletotrichum* spp. in only one of the three organs tested. In seven trees, all belonging to cultivar Galega vulgar, *Colletotrichum* spp. were found in more than one organ: in both the flower buds and immature fruits in three trees; in both the stems and immature fruits in three trees; and in all three organs in one tree.

3.3. Multivariate Data Analysis

The mean abundance $\pm$ standard error (SE) of the number of trees with the presence of *Colletotrichum* spp. was 2.77 $\pm$ 0.39 in Galega vulgar, followed by Azeiteira 1.73 $\pm$ 0.14 and Cobrançosa 1.63 $\pm$ 0.12, respectively. Galega vulgar showed significantly ($p = 0.0016$) higher values than Azeiteira and Cobrançosa (Figure 2).

The mean abundance $\pm$ SE of the olive trees with the presence of *Colletotrichum* spp. was 2.41 $\pm$ 0.47 in Elvas, followed by Monforte 2.19 $\pm$ 0.43 and Vidigueira 2.11 $\pm$ 0.39, respectively. No significant differences ($p > 0.05$) were shown between the sites (Figure 2). The mean abundance $\pm$ SE of the trees with *Colletotrichum* spp. was 2.71 $\pm$ 0.34 when the immature fruits were tested, followed by the 2-year stems 1.90 $\pm$ 0.27 and the bud flowers 1.42 $\pm$ 0.12, respectively (Figure 3). A significantly ($p = 0.0037$) higher number of trees with *Colletotrichum* spp. was found when the immature fruits were tested when compared with the 2-year stems and bud flowers.
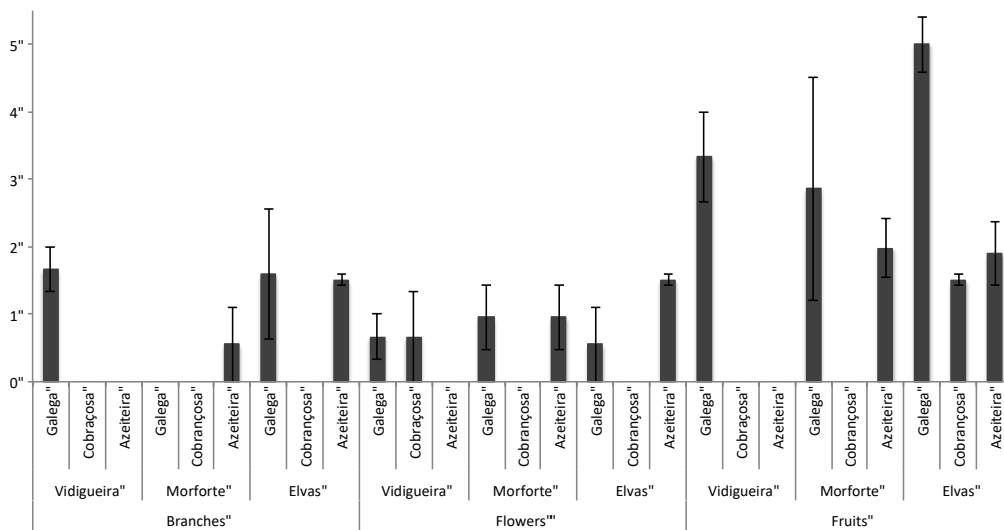
Figure 2. The mean number of trees with *Colletotrichum* spp. ± standard error (SE) after testing stems, bud flowers, and immature fruits of trees from each cultivar (Galega vulgar, Cobrançosa, and Azeiteira) in different sites (Vidigueira, Monforte, and Elvas).
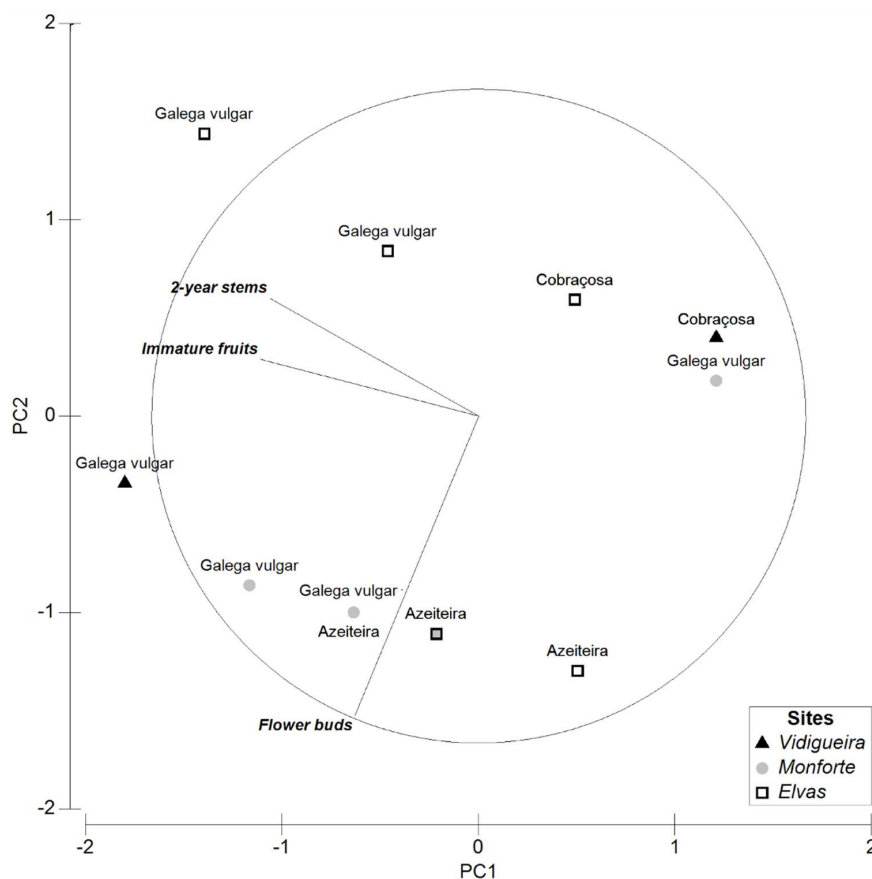

Figure 3. The principal component analysis (PCA) plot based on the variables measured: the 2-year stems, flower buds, and immature fruits in the a priori defined groups based on "Sites" (Monforte, Vidigueira, and Elvas) and "Cultivars" (Galega vulgar, Cobrançosa, and Azeiteira). PC1, 58.5%, and PC2, 29.5%, together accounted for 88.0% of the variability of the data.

Based on the variables measured—organs (2-year stems, bud flowers, and immature fruits), sites (Vidigueira, Monforte, and Elvas), and cultivars (Galega vulgar, Cobrançosa, and Azeiteira)—the PCA ordination showed that the first two components (PC1, 58.5% and PC2, 29.5%) together accounted

for 88.0% of the variability of the data. The PCA ordination clearly separated the samples of the immature fruits from the Galega vulgar cultivar at Elvas and Vidigueira sites mainly due to the fact that the highest values of *Colletotrichum* spp. were observed on this organ on this cultivar at these sites. The samples of the 2-year stems from cultivars Galega vulgar, Cobrançosa, and Azeiteira at Elvas were also clearly separated due to the high values of *Colletotrichum* spp. observed in those samples. In addition, the PCA ordination separated the samples with the lowest values or no *Colletotrichum* spp., such as the samples from the Cobrançosa and Azeiteira cultivars in Vidigueira and Cobrançosa in Monforte (Figure 3).

The SIMPER analysis showed how the presence of *Colletotrichum* spp. in the 2-year stems, flower buds, and immature fruits contributed to the similarity and dissimilarity of the a priori defined groups based on sites (Monforte, Vidigueira, and Elvas) and cultivars (Galega vulgar, Cobrançosa, and Azeiteira). In terms of the sites, the immature fruits showed the highest similarity, ranging from 49.15% in Vidigueira to 89.45% in Elvas, followed by the flower buds ranging from 9.65% in Elvas to 30.34% in Monforte and the 2-year stems ranging from 0% in Monforte to 24.57% in Vidigueira, respectively. The immature fruits showed the highest dissimilarities in terms of the sites, with values ranging from 47.79% (Vidigueira vs. Elvas) to 51.29% (Vidigueira vs. Monforte), followed by the 2-year stems with values ranging from 26.67% (Vidigueira vs. Elvas) to 29.05% (Monforte vs. Elvas) and the flower buds with values ranging from 20.7% (Monforte vs. Elvas) to 25.54% (Vidigueira vs. Elvas), respectively (Table 3a). In terms of cultivar, the immature fruits showed the highest similarity ranging from 61.75% in Azeiteira to 100.0% in Cobrançosa, followed by flower buds ranging from 0% in Cobrançosa to 38.25% in Azeiteira and the 2-year stems ranging from 0% in Cobrançosa and Azeiteira to 11.3% in Galega vulgar, respectively. The immature fruits also showed the highest dissimilarities in terms of cultivar, with values ranging from 35.57% (Cobrançosa vs. Azeiteira) to 59.18% (Galega vulgar vs. Cobrançosa) followed by the flower buds with values ranging from 18.0% (Galega vulgar vs. Azeiteira) to 51.55% (Cobrançosa vs. Azeiteira) and the 2-year stems with values ranging from 12.88% (Cobrançosa vs. Azeiteira) to 26.93% (Galega vulgar vs. Azeiteira), respectively (Table 3b).

Table 3. The contributions of the 2-year stems, flower buds, and immature fruits to the similarities and dissimilarities of the a priori defined groups; a. "Sites" (Monforte, Vidigueira, and Elvas) and b. "Cultivars" (Galega vulgar, Cobrançosa, and Azeiteira).

a.	Similarity %		
	46.8	63.3	56.1
Plant organ	Vidigueira	Monforte	Elvas
2-Year Stems	24.57	0.0	0.9
Flower Buds	26.28	30.34	9.65
Immature Fruits	49.15	69.66	89.45
	Dissimilarity %		
	44.0	52.5	38.7
Plant organ	Vidigueira vs. Monforte	Vidigueira vs. Elvas	Monforte vs. Elvas
2-Year Stems	27.43	26.67	29.05
Flower Buds	21.28	25.54	20.7
Immature Fruits	51.29	47.79	50.24
b.	Similarity %		
	68.7	47.4	73.0
Plant organ	Galega	Cobrançosa	Azeiteira
2-Year Stems	11.3	0.0	0.0
Flower Buds	9.34	0.0	38.25
Immature Fruits	79.36	100.0	61.75
	Dissimilarity %		
	64.4	43.3	43.5
Plant organ	Galega vs. Cobrançosa	Galega vs. Azeiteira	Cobrançosa vs. Azeiteira
2-Year Stems	22.4	26.93	12.88
Flower Buds	18.42	18	51.55
Immature Fruits	59.18	55.07	35.57

4. Discussion

Although the disease cycle of anthracnose has been studied for several years [2,26], some aspects are still not yet fully understood. It is known that the anthracnose pathogen infects several parts of the olive tree: buds, flowers, sepals, pedicels, peduncles, leaves, petioles, leaf scars, shoots, twigs, receptacles, and fruits [9]. However, there is lack of information about the presence of the pathogen in the interior of the plant organs, as well as the impact it may have on the initiation and development of the disease. In addition, there is not a clear relation between the *Colletotrichum* species identified (within the *C. acutatum* complex) and the olive cultivars with different degrees of susceptibility.

In this study, the presence of *Colletotrichum* spp. was determined in the interior of 2-year stems, flower buds, and immature fruits of anthracnose asymptomatic olive trees from three different cultivars with different susceptibilities to the pathogen, grown in three different sites in Alentejo, the largest olive producing region in Portugal. Initially, 68 *Colletotrichum* spp. isolates were selected for their morphological and cultural characteristics: the color of the colonies, which varied from pink to grey and orange and the shape of the conidia, which presented a fusiform shape [27–29]. The *ITS* regions from all 68 *Colletotrichum* isolates were first used for PCR and sequencing due to their easy amplification when compared to alternative genes; however, the analysis of *ITS*-rDNA resulted in sequences highly similar to five different *Colletotrichum* species, and the analysis was not able to discriminate among them; thus, β-tubulin and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) genes were amplified and sequenced, because both genes have previously shown good results in *Colletotrichum* species identification [6,20,30]. The results based on the single alignment and phylogenetic analysis of both the *β-tubulin* and *GAPDH* sequences, allowed for the categorization of all the isolates into two species (*C. nymphaeae* and *C. godetiae*) within the *C. acutatum* complex. The prevalence of the species belonging to the *C. acutatum* complex over the *Colletotrichum gloesporioides* complex, as observed here, has been associated with areas where anthracnose is endemic and more aggressive [4,10]. This was also shown in other studies performed in Spain, Italy, Tunisia, and in the Alentejo region (Portugal) where an incidence of 100% *C. acutatum* over *C. gloesporioides* was observed [2,9,10,27], associated with losses of over 90% [2].

To the best of our knowledge, this was the first time that the *C. godetiae* species was detected in Alentejo. A previous study revealed that all the isolates from the Alentejo region, were identified as *C. nymphaeae* [6]. Despite the low incidence of *C. godetiae* in Alentejo, as well as in Ribatejo (<6%) and Beira Baixa (<3%), this species showed high incidences in other Portuguese regions, showing predominance over other *Colletotrichum* species in the Trás-os-Montes region [6,10]. In other countries, such as Italy, Montenegro, and Greece, *C. godetiae* is the most frequent *Colletotrichum* species, leading some authors to suggest that this species is the most frequent olive anthracnose pathogen in the central Mediterranean [2,31]. These contrasting observations clearly suggest that environmental conditions could shape the population structure of olive anthracnose pathogens, and under unfavorable conditions to the disease, less virulent olive anthracnose pathogens, such as *C. godetiae*, may emerge. The lower virulence of *C. godetiae* when compared with *C. nymphaeae* and the low disease severity observed in the Alentejo region during the sampling year (field observations) may have created the opportunity for *C. godetiae* to appear. Nevertheless, the vast majority of the isolates were identified to be *C. nymphaeae* (95.6%), corroborating that this species is the key pathogen in olive anthracnose in Portugal, as observed in other countries [32,33].

All three cultivars showed the presence of *Colletotrichum* spp.; Galega vulgar showed a significantly higher number of infected trees and higher percentages of infected organs, followed by Azeiteira and Cobrançosa, respectively. This result could be explained by the strong susceptibility of Galega vulgar to the disease [10,11]. However, the relation between the presence of *Colletotrichum* spp. and the development of anthracnose is not linear. In fact, a higher presence of the *Colletotrichum acutatum* complex was found in Azeiteira when compared with Cobrançosa, despite Azeiteira being considered resistant to the disease and Cobrançosa moderately tolerant. This may mean that plants from the Azeiteira cultivar may present *Colletotrichum* spp. without developing the disease. However, it cannot be ignored

that information on the susceptibility of cultivars is sometimes discrepant due to factors such as the different ripeness times, as the susceptibility of fruit increases with ripeness, the misidentification of cultivars, the misidentification of the disease due to confusion with other pathogens that cause fruit rot, or the virulence of the pathogen populations [3,34]. The low susceptibility to anthracnose of both of the early maturing cultivars Azeiteira and Cobrançosa is probably because the fruits are usually collected before the conditions are optimal for the development of the disease. Late maturing cultivars are more affected than early maturing ones [35,36], which may also explain why Galega vulgar, a late maturing cultivar, is so susceptible to anthracnose. In addition, Galega vulgar, in contrast with Azeiteira and Cobrançosa, is very susceptible to the olive fly, which contributes to the existence of wounds that are, in turn, related to the increase of the rate of colonization of the fungus and the severity of the symptoms [1,37,38]. The thinner epidermis cells of the Galega vulgar fruits, when compared with other less susceptible cultivars, also provide lower protection against pathogens [39].

All the sites showed the presence of *Colletotrichum* spp. Elvas showed the highest number of infected trees, followed by Monforte and Vidigueira, respectively; however, these differences were not significant. Similarities in the environmental conditions and the management at the different sites may have contributed to these small differences.

All three of the tested plant organs presented *Colletotrichum* spp. in their interior. The immature fruits showed a significantly higher presence of *Colletotrichum* spp., followed by the flower buds and the 2-year stems, respectively. These results differ from the ones obtained by Moral et al. [9], who showed that developing fruits were the plant organs that showed the lowest percentages of *Colletotrichum* spp. (<1.5%) when compared with stems and flowers—very different from the 12.6% (34 trees with *Colletotrichum* spp. in their fruits out of the 270 olive trees tested) obtained in this study. The sampling was performed following the dry spring of 2016, and the pathogen inoculum might have been even higher if the sampling was performed after a rainy season, as periodic rain events in the spring lead to high levels of the presence of the pathogen on vegetative organs [6]. In addition, the severity of the disease in autumn is not correlated with the level of presence of *Colletotrichum* spp. in vegetative organs but, instead, with the weather conditions in autumn.

Overall, our results suggest that the olive tree may serve as important source of inoculum. In addition, in our survey, no mummified fruits were observed, suggesting that they are not an essential source of inoculum, as already shown by other authors [6]. The inoculum present inside the symptomless organs tested in this study may be responsible for the primary infection of *Colletotrichum* spp. together with the latent infection of flowers and young fruits in the spring and summer, respectively, as suggested by Moral et al. [9]. Trees become infected through the invasion of the mycelium from wounds or the peduncles and the petioles of the affected fruits and leaves, respectively [1]; however, it had not been confirmed previously whether the fungus moves inside the plant, infecting other parts of the plant. It was interesting to verify that *C. nymphaeae* 2, characterized by a unique nucleotide mutation within the beta tubulin gene, was found in different organs of the same tree—in the 2-year stems and in recently formed vegetative organs, such as flower buds and immature fruits—which seem to suggest that the infection may be caused the same isolate, which has moved systemically inside of the plant. In addition, *C. godetiae* was shown to be rare, but it was found in both the flower buds and fruits of the same tree.

Interestingly, *Colletotrichum* spp. was found simultaneously in different organs in seven trees, all of them belonging to cultivar Galega vulgar; this may help us to understand the high susceptibility of the cultivar, which may be associated with the greater ability of the fungus to move inside the plant.

All these observations, together with the fact that the highest percentages of the infected immature fruits were obtained in trees that also presented a high percentage of 2-year stem infections, seem to support the idea of the systemic movement of *Colletotrichum* spp. inside olive trees.

5. Conclusions

The present study shows that the majority of *Colletotrichum* spp. isolated from olive trees in Alentejo, Portugal, belongs to *C. nymphaeae* and, for the first time, *C. godetiae* was detected in Alentejo region. The highest number of *Colletotrichum* spp. isolates was detected in immature fruits in trees from cv. Galega vulgar. The highest percentages of infected immature fruits were obtained in trees that also presented a high percentage of 2-year stem infections, which may indicate that the fungus may travel from the stems to other parts of the plant. Another indication of systemic movement is that one isolate of *C. nymphaeae* was present in different organs of the same tree. The recognition of a systemic phase in *Colletotrichum* spp. in olive trees would change our understanding of anthracnose and would have relevant implications on choosing the best control strategies. In fact, such a discovery may help to explain why the largely use of contact copper-based fungicides is not successful in controlling the disease under favorable conditions, as such fungicides only destroy the epiphytic pathogens and only prevent new infections from the outside. The application of a systemic fungicide in the late winter or early spring to eliminate/reduce latent infections may be essential to reduce the incidence of the disease. The results presented here can play an important role in developing strategies for the effective and timely management of the disease and in reducing the number of unnecessary fungicide applications.

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