

Article

Diversity of Endophytic and Pathogenic Fungi of Saffron (*Crocus sativus*) Plants from Cultivation Sites in Italy

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Abstract: *Crocus sativus* is an important crop for the production of saffron and bioactive compounds. Plant endophytic fungi are a source of secondary metabolites additional to those produced by the plant itself. We analysed the biodiversity of endophytic fungi present in corms, stems, leaves, tepals, and stigmas of *C. sativus* from ten Italian sites; furthermore, we isolated putative pathogenic fungi from rotten plants. We used an in vitro isolation approach followed by molecular analysis of the internal transcribed spacer (ITS rDNA) region. We obtained 165 strains belonging to 39 OTUs, spreading over 26 genera and 29 species. Dark septate endophytes of the genus *Cadophora* and the species *Talaromyces pinophilus* dominated in corms, while *Alternaria alternata*, *Epicoccum* spp., *T. pinophilus*, *Mucor fragilis*, and *Stemphylium vesicarium* dominated in other tissues. The most frequently isolated pathogens were *Fusarium oxysporum* and *Rhizopus oryzae*. Endophytic communities significantly differed among tissues and life stages, whereas differences among cultivation sites were not statistically supported. Several endophytes were hypothesized to have changing trophic modes and/or to be latent pathogens in *C. sativus*. All strains were conserved ex-situ for future bioactivity tests and production of metabolites.

Keywords: *Cadophora* spp.; corm rot; dark septate endophytes; functional guild; internal transcribed spacer; *Talaromyces* spp.



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1. Introduction

Endophytic fungi are defined as fungi colonizing the intercellular spaces of living, healthy plant tissues, without triggering disease symptoms [1–3]. Rather, they are mutualists providing their hosts with resistance to biotic and abiotic stresses and receiving protection and nutrients in exchange from the plant. The finding of the anticancer drug paclitaxel (Taxol) from the endophytic fungus *Taxomyces andreanae* with the host species *Taxus brevifolia* [4] inspired the search and study of endophytic fungi from various host plants [5,6]. Many studies have explored the diversity and biotechnological potential of endophytic fungi across the most diverse plant species and tissues in different ecological niches [3,7]. Particular attention has been paid to the endophytic fungal diversity of crop plants and the potential use of these fungi as biocontrol agents for the management of plant diseases with a low impact to the environment as they allow the reduction in agrochemicals and fertilizers [8].

Crocus is a plant genus in the family Iridaceae. It comprises about 85 herbaceous species distributed between the Mediterranean, Europe, and Western Asia [9]. *Crocus sativus* L. (saffron plant) is a sterile triploid plant propagated as a clonal lineage using corms and is the most economically important species in this family [10]. The evolutionary origin of saffron has been debated for almost a century [11]. According to the most recent studies, this crop evolved in Attica (Greece) by the combination of two genotypes of its closest relative *Crocus cartwrightianus* [11,12]. The aromatic properties, bitterness and natural colouring of its dried stigmas make it the most expensive spice in the world, also because

of the intense labour and time required for its cultivation and manual harvesting. Stigmas have been used since ancient times in several sectors: food, dyeing, perfumery, cosmetics, and medicine [13]. Nowadays, *C. sativus* is cultivated successfully under different environmental conditions in several countries of Asia (e.g., Iran, India) and in the Mediterranean basin, e.g., Greece, Morocco, Spain, and Italy [14]. In Italy, saffron is traditionally cultivated in Navelli (Abruzzo region), and in S. Gavino Monreale (Sardinia), but, in the last two decades, the cultivation has spread all over the country.

However, the lack of modern approaches to cultivation (mostly executed by hand), the spice's expensiveness, and frequent adulteration with other products (such as pomegranate fruit peel, safflower, or tumeric [15]) has resulted in a decline in saffron cultivation worldwide. Moreover, corm rot due to nematodes and fungi (*Fusarium*, *Penicillium*, *Rhizoctonia*, etc.) is frequent in cultivation sites causing significant crop losses [14]. Exploring the plant–endophyte interactions in saffron may represent a good approach to start adopting scientific practices to cultivate this species sustainably. In fact, several endophytic species produce antibiotics and antifungal compounds that protect plants against pathogenic nematodes, insects, bacteria and fungi, holding promises for eco-friendly and economically sustainable agriculture [16].

To the best of our knowledge, only a few studies for the identification of saffron endophytic fungi have been conducted, mostly from the belowground tissues of some cultivation sites in India and Morocco [17–21]. In parallel, soil fungal communities have been characterized in some saffron cultivation sites [19,22]. Exploring the endophytic fungal communities of saffron plants in different tissues besides the corm, such as tepals and stigmas, would be of particular interest since the species composition of the endophytic community may change across tissues depending on the ability of the endophytic species to use specific substrates [23]. In addition, *in vitro* activities against phytopathogenic fungi of saffron stigmas have been reported [24,25], and the involvement of endophytic fungi in the production of the related bioactive molecules cannot be ruled out.

The objectives of our research were to characterize and estimate the diversity of the fungal endophytes associated with different tissues (corms, stems, leaves, tepals, and stigmas) of *C. sativus* cultivated in different sites of the Umbria region (central Italy) and in Sicily (south Italy). We adopted an isolation-based approach to build a strain collection to be used in future screening for the identification of biologically active molecules and biocontrol agents against plant pathogens. To this purpose, several pathogenic fungal strains were also isolated and identified from rotten saffron plants, as potential targets for interaction studies.

This study is the first report of endophytic fungi associated with *C. sativus* in the Mediterranean basin and the first examining tepals and stems of the saffron plant.

2. Materials and Methods

2.1. Biological Material and Study Sites

Crocus sativus healthy plants were collected in the years 2017–2019 from ten Italian cultivation sites, nine in the Umbria region and one in Sicily (Table 1). Geographical distances varied between 7 and 77 km between sampling sites of the Umbria region, whereas the Sicily site was more than 600 km away from all the others (Figure S1). A total of 73 individual plants were collected. However, depending on the harvesting life stage, not all tissues could be collected from each plant. For example, tepals and stigmas were collected in the flowering stage only, whereas corm samples, being a perennial part of the plant, were sampled at all stages of the plant life cycle. Therefore, a total of 50 corms, 8 stems, 18 leaves, and 18 flowers (tepals and stigmas) were sampled. Overall, three growth stages were sampled, the vegetative (March to May), the dormant (August), and the flowering (October to November). Additionally, six rotten plants were collected in 2018 from one site (Moiano) and used to isolate putative pathogenic fungi from corms, stems, and leaves (Table 1).

Table 1. Sampling sites, life stages, and health state of the examined saffron tissues. * Samples collected from rotten plants; ** life stages: v= vegetative, f = flowering, d = dormant.

Sampling Site	Locality	Latitude	Longitude	Altitude (m)	Sampled Tissues *	No. of Samples	Life Stage **
1	S. Martino in Colle (Umbria)	43.0335354	12.3644377	275	corm	7	v
					leaf	7	v
					tepala	3	f
					stigma	3	f
2	Città della Pieve (Umbria)	42.9527338	12.004326	513	leaf	5	v
					3		
3	Moiano (Umbria)	43.0148483	12.0184455	268	corm	6	v
					corm	5	f
					corm *	6	f
					leaf	6	v
					leaf *	6	f
					stem	8	f
					stem *	6	f
					tepala	3	f
					stigma	3	f
					4	Gualdo Cattaneo (Umbria)	42.9094087
5	Giano dell'Umbria (Umbria)	42.8334672	12.5777111	542	tepala	3	f
					corm	4	v
6	Castel Ritaldi (Umbria)	42.8232601	12.6722871	297	corm	5	d
					corm	3	f
					tepala	3	f
					stigma	3	f
					tepala	3	f
					7	Foligno (Umbria)	42.9561825
8	Cantalupo di Bevagna (Umbria)	42.9683821	12.5796837	201	corm	4	v
					corm	5	d
					corm	3	f
					tepala	3	f
					stigma	3	f
9	Città di Castello (Umbria)	43.4566183	12.3247772	566	corm	2	d
					3		
10	Zafferana Etnea (Sicily)	37.6932846	15.1064599	584	corm	2	v

2.2. Isolation of Fungi and Molecular Identification

The corms were washed under tap water after removing the fibrous external layer. All tissues were surface sterilized as described by Wani et al. [18] with some modifications: 0.3% sodium hypochlorite for 5 min (corms) or 3 min (other tissues), followed by 70% ethanol for 2 min (corms) or 1 min (other tissues), and they were finally rinsed three times with sterile distilled water and allowed to surface dry under sterile conditions. The different tissues (inner tissue for the corms) were cut into 0.5–1 cm segments with a sterile surgical blade and placed on potato dextrose agar (PDA, Merk Life Science S.r.l., Milano, Italy) added with 100 mM ampicillin to avoid bacterial contamination. For each corm, stem, leaf, and tepal sample, 10 tissue segments were taken, whereas 4 segments were taken for each stigma. Cultures were incubated at 25 °C (corm cultures in the dark) and checked for hyphae growing out of the tissues every 3–4 days, up to 4 weeks. For each tissue sample, all the mycelia with different morphological features were picked and re-inoculated onto fresh PDA in Petri dishes to obtain pure cultures. Finally, the cultures were transferred in potato dextrose broth (PDB, Merk Life Science S.r.l., Milano, Italy) with 50% (*v/v*) glycerol, frozen in liquid nitrogen and stored long-term at −70 °C. To isolate fungal strains from rotten plants, the same procedure was adopted but avoiding the preliminary surface sterilization of tissues.

Genomic DNA was isolated from each strain as described in [26]. Briefly, about 0.3 g of mycelium was crushed and suspended in 300 µL of buffer containing 200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, and 0.5% SDS, vortexed for 10 s, and centrifuged for 10 min at 14,000 rpm. The supernatants were precipitated in an equal volume of

isopropanol for 30 min at -20°C . The DNA was pelleted by centrifugation for 20 min, vacuum-dried, and resuspended in 100 μL of double-distilled nuclease-free water. DNA concentration was determined using a NanoDrop 2000 UV-vis Spectrophotometer (Thermo Scientific). The full ITS region was PCR amplified with the primers ITS1f [27] and ITS4 [28]. PCRs were carried out in a 25 μL reaction mixture containing template DNA (10 ng), $10\times$ PCR buffer (GE Healthcare, Life Sciences, Marlborough, MA, USA) 4 mM MgCl_2 , dNTPs (0.2 mM each), 10 μM of each primer, and 1 U of Taq polymerase (GE Healthcare Life Sciences). A GeneAmp[®] PCR System 9700 (Applied Biosystems, Foster City, CA, USA) was used to perform PCRs under the following conditions: initial denaturation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 20 s, extension at 72°C for 45 s, and a final extension at 72°C for 7 min. Sequencing was conducted using the primers ITS1f, ITS4, 5.8sf, and 5.8sb [28,29] and the BigDye Terminator Cycle V 3.1 Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the supplier's instructions. Capillary electrophoresis was carried out with an ABI 3130 Genetic Analyzer (Applied Biosystems). Electropherograms were analysed with FINCHTV v. 1.3.1 (Geospiza, Inc., Seattle, WA, USA; <http://www.geospiza.com>, accessed on 20 January 2020). The resulting DNA sequences were deposited in GenBank, and accession numbers are provided in Table 2.

Table 2. Description of the OTUs representing the fungal endophytes and pathogens isolated from different tissues of *Crocus sativus*.

OTU		Closest Match in GenBank			No. of Isolates					
Name	Accession No.	Taxon	Accession No.	% of Similarity	Corms	Stems	Leaves	Tepals	Stigmas	Total
ASCOMYCOTA										
Helotiales										
1	MW798781	<i>Cadophora luteo-olivacea</i>	HM116747	99.8	32					32
2	MW798782	<i>Cadophora malorum</i>	KF646089	99.8	19					19
3	MW798777	<i>Botrytis cinerea</i>	MH860108	100			1			1
Pleosporales										
4	MW798757	<i>Alternaria alternata</i>	MT453271	100		1	1	13	4	19
5	MW798753	<i>Alternaria infectoria</i>	MK461063	99.8				1		1
6	MW798751	<i>Stemphylium vesicarium</i>	MK461018	100			4			4
7	MW798765	<i>Pyrenophora tritici-repentis</i>	MH399396	99.8				1		1
8	MW798750	<i>Epicoccum</i> sp.	HQ630972	100			7			7
36	MW798766	<i>Epicoccum nigrum</i>	MH931271	98.4		(1)				(1)
9	MW798760	<i>Stagonosporopsis cucurbitacearum</i>	KM489071	98.6				1		1
10	MW798754	<i>Spegazzinia</i> sp.	KR093917	99.1					1	1
Dothideales										
11	MW798756	<i>Aureobasidium pullulans</i>	FN868454	99.2			(1)	2	1	3 (1)
12	MW798769	<i>Aureobasidium pullulans</i>	MT153709	95.64		1				1
Capnodiales										
13	MW798749	<i>Cladosporium cladosporioides</i>	MH863979	100			1			1
Eurotiales										
14	MW798764	<i>Talaromyces pinophilus</i>	KC867288	99.7	7 (2)	2				9 (2)
15	MW798762	<i>Talaromyces cecidicola</i>	MH862736	99.8	5	1				6
16	MW798752	<i>Talaromyces assiutensis</i>	JN899320	99.5	1		1			2
17	MW798763	<i>Aspergillus niger</i>	MF422165	100	2					2
18	MW798758	<i>Aspergillus</i> sp.	MK461022	100					1	1
19	MW798755	<i>Aspergillus flavipes</i>	HM595494	99.4					1	1
20	MW798761	<i>Aspergillus europaeus</i>	LT220221	99.7		1				1
21	MW798759	<i>Penicillium citrinum</i>	KX958075	99.8		1				1
Hypocreales										
22	MW798779	<i>Fusarium oxysporum</i>	MT453296	100	1 (9)	(3)	(1)		1	2 (13)
23	MW798774	<i>Ilyonectria</i> sp.	KT268970	100	1					1
24	MW798780	<i>Parengyodontium album</i>	LC092887	99.6	1					1
35	MW798772	<i>Trichoderma</i> sp.	MW450867	99.8			(1)			(1)
Xylariales										
25	MW798776	<i>Hypoxylon fuscum</i>	MW367856	99.6			1			1
Sordariales										
26	MW798778	<i>Ovatospora brasiliensis</i>	MH858514	99.8					1	1

Table 2. Cont.

OTU		Closest Match in GenBank			No. of Isolates					
Name	Accession No.	Taxon	Accession No.	% of Similarity	Corms	Stems	Leaves	Tepals	Stigmas	Total
27	MW798775	Leotiomyces incertae sedis <i>Malbranchea circinata</i>	MN627784	99.4			1			1
28		Saccharomycetales <i>Meyerozyma caribbica</i>	KY104217	100	1	1				2
29	MW798748	BASIDIOMYCOTA Sporidiobolales <i>Rhodotorula</i> sp.	HG936596	99.7	1	1		1		3
30	MW798746	Agaricales <i>Coprinellus micaceus</i>	FN386285	99.8			1			1
31	MW798745	Russulales <i>Peniophora</i> sp.	MT156128	99.5		1				1
32	MW798744	Filobasidiales <i>Filobasidium wieringae</i>	KY103450	100				1		1
34	MW798747	uncultured fungus/ <i>Filobasidium</i> sp.	AF444450	99.7			1			1
33	MW798768	MUCOROMYCOTA Mucorales <i>Mucor fragilis</i>	KU319073	100				2	3	5
37	MW798770	<i>Mucor circinelloides</i>	KP132468	99.8	(1)					(1)
38	MW798773	<i>Rhizopus oryzae</i>	MF685318	99.9	(5)	(2)				(7)
39	MW798771	<i>Rhizopus oryzae</i>	HQ435056	98	(3)		(1)			(4)
Total No. isolates					71 (20)	10 (6)	19 (4)	22	13	135 (30)
Species richness					11 (5)	9 (3)	10 (4)	8	8	34 (8)

In parentheses, the number of strains isolated from rotten plants are indicated.

Assembly, editing, and alignment of sequences were conducted using BIOEDIT v.7.2.5 [30]. Similarity searches were performed both in GenBank and UNITE databases using BLASTn [31]. In order to designate operational taxonomic units (OTUs), sequences were clustered using a 97% similarity threshold using CD-HIT-EST [32] (<http://weizhong-lab.ucsd.edu/cdhit-web-server/cgi-bin/index.cgi?cmd=cd-hit-est>, accessed on 15 June 2020).

2.3. Phylogenetic Analysis

Two independent multiple sequence alignments were performed for Ascomycota and for Basidiomycota plus Mucoromycota, respectively. Phylogenetic analysis was performed using the maximum-likelihood (ML) method using RaxML version 8.2.12 [33] using the CIPRES Science Gateway web service (<https://www.phylo.org/portal2/login>, accessed on 10 February 2021). The analysis was performed using rapid bootstrapping, the GTRGAMMA distribution model, and empirical base frequency options. *Candida* (Saccharomycotina) and *Rhizopus* (Mucoromycotina) were used as the outgroups. Phylogenetic trees were visualized with Figtree version 1.4.4 (<https://github.com/rambaut/figtree/releases>, accessed on 10 February 2021) and further processed with Inkscape version 0.91 (<https://inkscape.org/>, accessed on 20 November 2016). Alignments and trees were submitted to TreeBASE (S28273).

2.4. Diversity Analyses

The alpha diversity was measured as species richness and was calculated for each tissue, for the different life stages of corms and for the four main sampling sites. Since different numbers of corms, stems, leaves, and flowers were sampled, and from different plants, we could not calculate the OTU's relative abundance per plant. However, the OTU's relative abundance was calculated for every single tissue (number of isolates of a given OTU in a tissue/number of total isolates of that tissue). The dominant species in each tissue were calculated according to Rivera-Orduña et al. [34] as those OTUs with $P_i > 1/S$, being species richness (S), the number of OTUs in the different tissues and P_i , and the relative abundance, i.e., the ratio number of isolates of one species/total isolates.

To evaluate beta diversity, the presence–absence dissimilarity indices of Jaccard [35] and Sorensen [36], and principal coordinate analysis (PCoA) were calculated using R v. 3.6.2, using the package VEGAN v. 2.5.6 (<https://cran.r-project.org/web/packages/vegan/index.html>, accessed on 25 October 2020). Hierarchical clustering was performed using the R function HCLUST using the “complete” agglomeration method and the Jaccard dissimilarity values as input. To test for statistical differences, permutational analysis of variance (PERMANOVA) [37] was calculated with 10,000 permutations, using the adonis function of the vegan package. Statistical tests were considered significant at $p < 0.05$.

To make functional guild annotations of the fungal community, taxonomic assignments were compared at the genus level to the FUNGuild database [38].

3. Results

3.1. Isolation and Identification of Saffron Endophytic Fungi

A total of 135 fungal isolates were recovered from corms (72), stems (10), leaves (18), tepals (22), and stigmas (13) of *C. sativus* healthy plants. Additionally, 30 fungal isolates were obtained from corms (20), stems (6), and leaves (4) of rotten saffron plants.

The isolates were identified by means of the full ITS ribosomal gene sequence analyses. Clustering of the sequences at 97% of identity allowed the detection of 39 OTUs (Table 2). The putative species names, inferred by BLASTn searches and phylogenetic analysis (Figures 1 and 2), are reported in Table 2.

More specifically, 30 OTUs belonged to Ascomycota, 5 to Basidiomycota, and 4 to Mucoromycota. Most of the OTUs belonging to Ascomycota were Pezizomycotina and clustered in four classes; Dothideomycetes were the most represented (11 OTUs, 1 from rotten plants), followed by Eurotiomycetes (8 OTUs), Sordariomycetes (6 OTUs, 1 from rotten plants), and Leotiomycetes (4 OTUs). Saccharomycotina was represented by 1 OTU only, in the class Saccharomycetes. Among Ascomycota, the orders with the highest number of OTUs were Eurotiales (8 OTUs) and Pleosporales (8 OTUs, 1 from rotten plants), followed by Hypocreales (4 OTUs, 2 from rotten plants), Helotiales (3 OTUs), Dothideales (2 OTUs), and Capnodiales, Xylariales, Sordariales, Leotiomycetes incertae sedis, and Saccharomycetales (1 OTU each) (Figure 1, Table 2). Most of the Basidiomycota OTUs belonged to the Agaricomycotina, in the classes of Agaricomycetes (2 OTUs) and Tremellomycetes (2 OTUs), whereas only one OTU was clustered in the Pucciniomycotina, in the class Microbotryomycetes. Among Basidiomycota, four orders were represented, namely, Sporidiobolales, Agaricales, Russulales, and Filobasidiales (1 OTU each). Finally, all the Mucoromycota (4 OTUs, 3 from rotten plants) belonged to the class Mucoromycetes, order Mucorales.

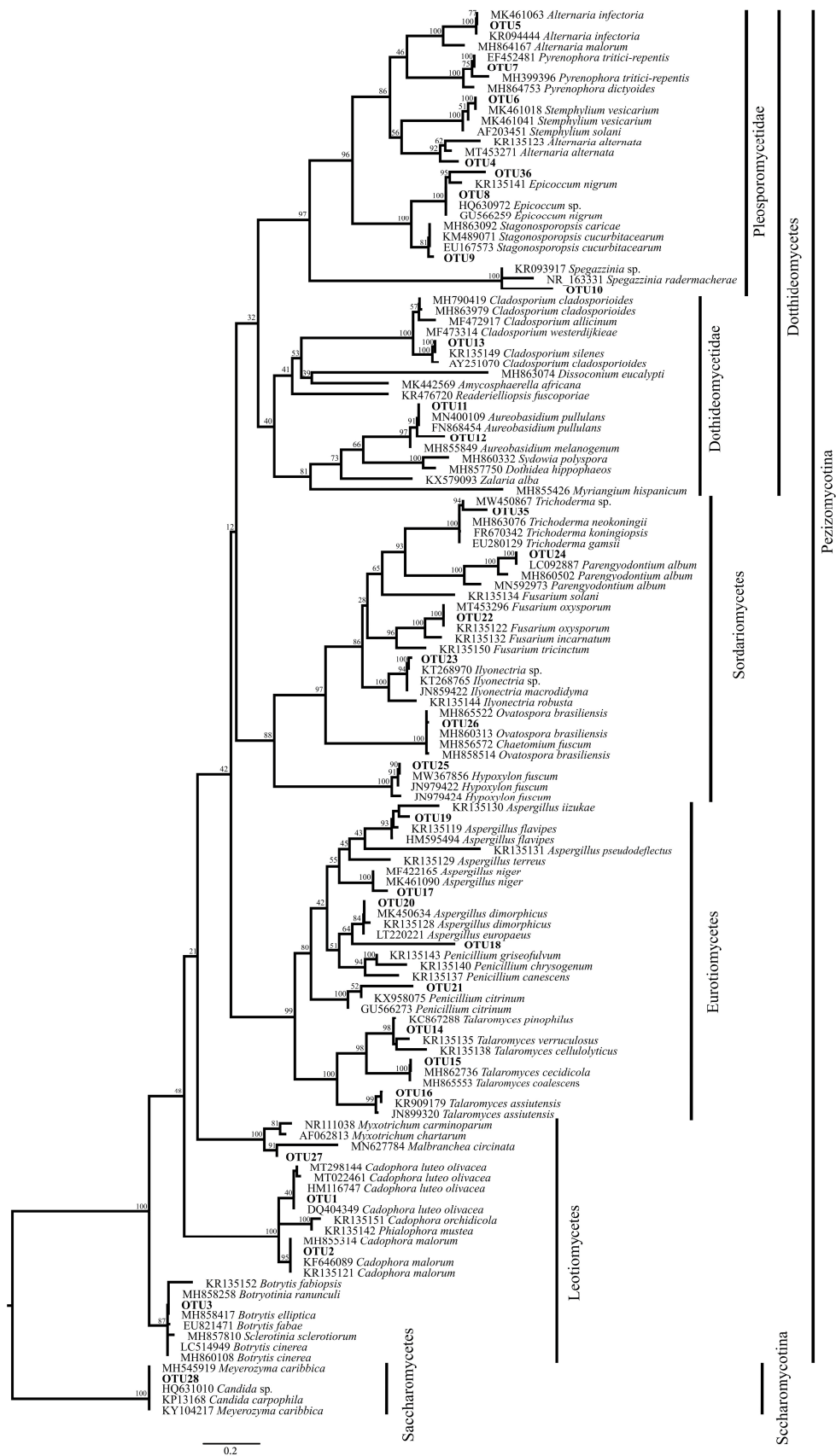


Figure 1. Phylogenetic tree of the Ascomycetes OTUs identified in this study (in bold). Numbers near the branches indicate bootstrap values (percentage over 1000 replicates).

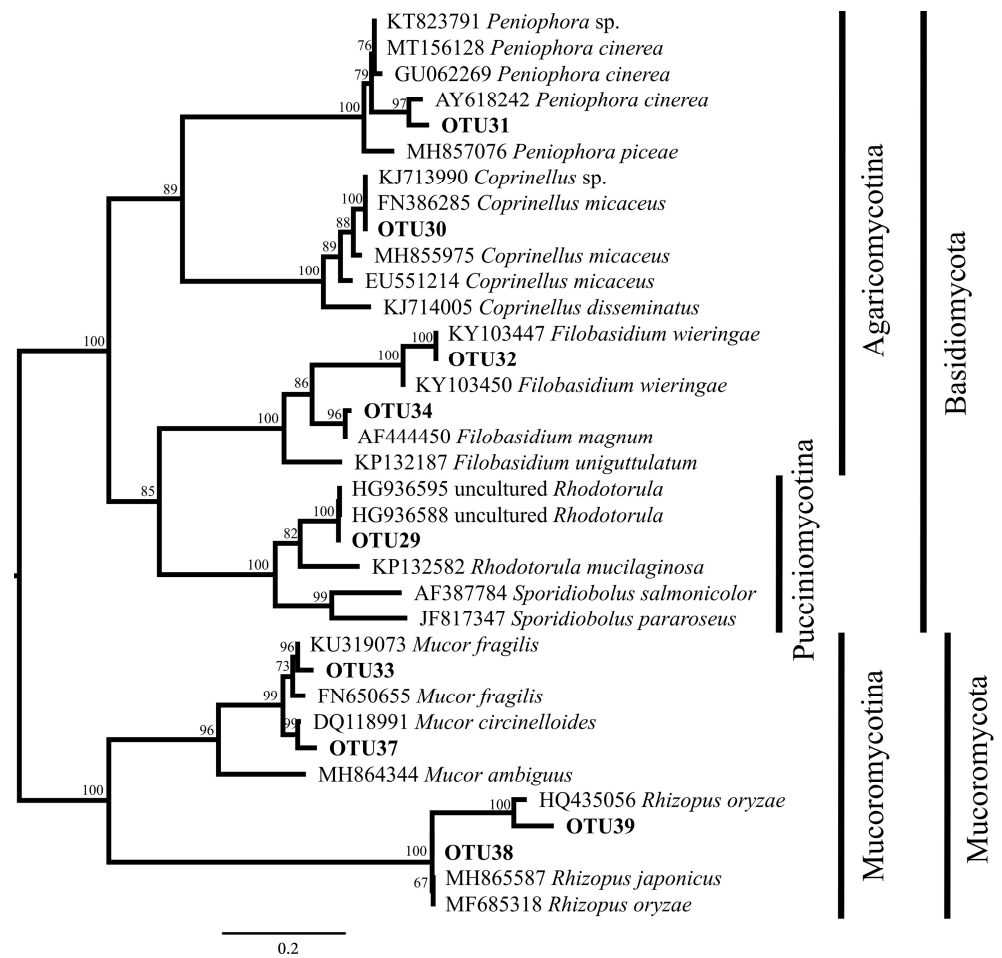


Figure 2. Phylogenetic tree of Basidiomycota and Mucoromycota OTUs identified in this study (in bold). Numbers near the branches indicate bootstrap values (percentage over 1000 replicates).

3.2. Endophytic Fungi from Healthy Plants

A total of 34 OTUs were identified among the fungi isolated from healthy plants. Using BLASTn and phylogenetic analyses, species names were assigned to 25 OTUs, while nine OTUs were identified at the genus level only. The Ascomycota were the most represented (82.3%, 28 OTUs), whereas Basidiomycota (14.7%, 5 OTUs) and Mucoromycota (2.9%, 1 OTU) were only a few and with a small number of isolates (Table 2).

Species richness (S) estimation showed a uniform alpha diversity in the different plant tissues, being S values ranging from 8 to 11, despite the different number of isolates collected from each tissue (Table 2). Some OTUs, corresponding to *Alternaria alternata*, *Aureobasidium pullulans*, *Talaromyces* spp., and *Fusarium oxysporum* were shared between different tissues.

Dominant species were identified in each tissue as those species with $P_i > 1/S$ (see Material and Methods and Table 3).

Table 3. Relative abundance of the OTUs and dominant fungal species in the different tissues of *C. sativus* healthy plants.

OTU	Taxon	Pi				
		Corms	Stems	Leaves	Tepals	Stigmas
1	<i>Cadophora luteo-olivacea</i>	0.451 *				
2	<i>Cadophora malorum</i>	0.268 *				
3	<i>Botrytis cinerea</i>			0.053		
4	<i>Alternaria alternata</i>		0.1	0.053	0.591 *	0.308 *
5	<i>Alternaria infectoria</i>				0.045	
6	<i>Stemphylium vesicarium</i>			0.211 *		
7	<i>Pyrenophora tritici-repentis</i>				0.045	
8	<i>Epicoccum</i> sp.			0.368 *		
9	<i>Stagonosporopsis cucurbitacearum</i>				0.045	
10	<i>Spegazzinia</i> sp.					0.077
11	<i>Aureobasidium pullulans</i>				0.091	0.077
12	<i>Aureobasidium pullulans</i>		0.1			
13	<i>Cladosporium cladosporioides</i>			0.053		
14	<i>Talaromyces pinophilus</i>	0.099 *	0.2 *			
15	<i>Talaromyces cecidicola</i>	0.070	0.1			
16	<i>Talaromyces assiutensis</i>	0.014		0.053		
17	<i>Aspergillus niger</i>	0.028				
18	<i>Aspergillus</i> sp.					0.077
19	<i>Aspergillus flavipes</i>					0.077
20	<i>Aspergillus europaeus</i>		0.1			
21	<i>Penicillium citrinum</i>		0.1			
22	<i>Fusarium oxysporum</i>	0.014				0.077
23	<i>Ilyonectria</i> sp.	0.014				
24	<i>Parengyodontium album</i>	0.014				
25	<i>Hypoxylon fuscum</i>			0.053		
26	<i>Ovatospora brasiliensis</i>					0.077
27	<i>Malbranchea circinata</i>			0.053		
28	<i>Meyerozyma caribbica</i>	0.014	0.1			
29	<i>Rhodotorula</i> sp.	0.014	0.1		0.045	
30	<i>Coprinnellus micaceus</i>			0.053		
31	<i>Peniophora</i> sp.		0.1			
32	<i>Filobasidium wieringae</i>				0.045	
34	Uncultured fungus/ <i>Filobasidium</i> sp.			0.053		
33	<i>Mucor fragilis</i>				0.091	0.231 *
	1/S	0.091	0.111	0.1	0.125	0.125

Pi = ratio number of isolates of one species/total isolates. * Dominant species (Pi > 1/S).

In the corms, the 1/S value was 0.091, and a strong dominance of *Cadophora luteo-olivacea* (OTU1) was found (Pi = 0.451), followed by *Cadophora malorum* (OTU2, Pi = 0.268) and *Talaromyces pinophilus* (OTU14, Pi = 0.099). *Cadophora* spp. were detected in corms only, whereas *T. pinophilus* also occurred in stems. *Epicoccum* (OTU8) and *Stemphylium vesicarium* (OTU6) dominated in leaves and *Alternaria alternata* (OTU4) in tepals. In stigmas, a slight dominance of *A. alternata* (OTU4) and *Mucor fragilis* (OTU33) was observed. *A. alternata* occurred in all tissues except corms (Table 3).

Beta-diversity analysis showed that the different tissues were distributed in three groups according to their similarity; a group was constituted by tepals and stigmas, the second by corms and stems, and the third by leaves only (Figure 3a; Table S1a). A similar grouping was evidenced by the PCoA (Figure S2). Differences were statistically significant (PERMANOVA, $F = 3.5418$, $p = 0.00009$). Clustering based on Sorensen's dissimilarity gave us similar results (data not shown).

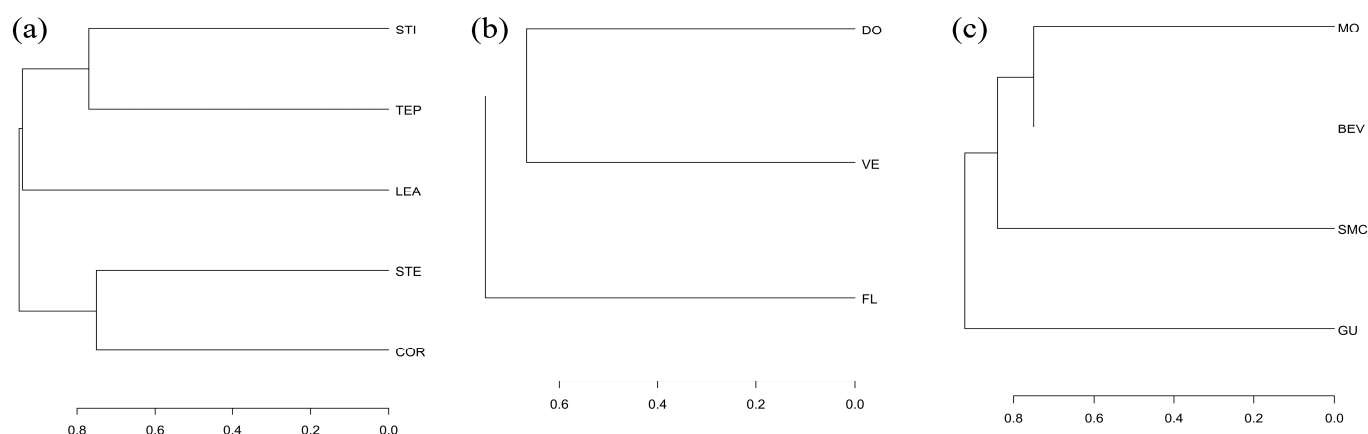


Figure 3. Cluster analysis, based on Jaccard dissimilarity values, of different tissues (a), life stages (b), and sites (c). STI = stigmas, TEP = tepals, LEA = leaves, STE = stems, COR = corms, DO = dormant, VE = vegetative, FL = flowering, MO = Moiano, BEV = Cantalupo di Bevagna, SMC = S. Martino in Colle, and GU = Gualdo Cattaneo.

Since fungal strains were isolated from all corm samples, we could evaluate the colonization levels and species diversity in corm tissue in different life stages. In the vegetative and flowering stages, a similar number of isolates were obtained (30 and 31, respectively), whereas ten isolates were obtained from the dormant stage (Table 4). The species richness was similar in the vegetative and flowering stages (six and eight OTUs, respectively), which shared the two genera *Cadophora* and *Talaromyces*. A lower species richness (two OTUs) was found in the dormant stage, where *Talaromyces* spp. and the other species were not found. *Cadophora luteo-olivacea* and *Cadophora malorum* occurred in corms at all life stages (Table 4).

Table 4. Diversity of fungal endophytes in corms of *C. sativus* at the vegetative, dormant, and flowering stages.

OTU	Taxon	Life Stage		
		Vegetative	Dormant	Flowering
1	<i>Cadophora luteo-olivacea</i>	12	6	14
2	<i>Cadophora malorum</i>	12	4	3
14	<i>Talaromyces pinophilus</i>	3		4
15	<i>Talaromyces cecidicola</i>			5
16	<i>Talaromyces assiutensis</i>	1		
17	<i>Aspergillus</i> sp.			2
22	<i>Fusarium oxysporum</i>			1
23	<i>Ilyonectria</i> sp.	1		
24	<i>Parengyodontium album</i>			1
28	<i>Meyerozyma caribbica</i>			1
29	<i>Rhodotorula</i> sp.	1		
Total No. isolates		30	10	31
Species richness		6	2	8

Beta-diversity analyses showed a slightly higher similarity among the vegetative and the dormant stages with respect to similarities among these stages and the flowering stage (Table S1b; Figure 3b). Differences were statistically significant (PERMANOVA, $F = 2.1453$, $p = 0.0028$). Clustering based on Sorensen's dissimilarity gave us similar results (data not shown).

Considering the four main localities, with the highest number of saffron tissues and fungal isolates collected, the highest alpha-diversity (species richness) was found in Moiano (20 OTUs), followed by Bevagna (10 OTUs), S. Martino in Colle (9 OTUs), and Giano dell'Umbria (5 OTUs) (Table 5).

Table 5. Diversity of fungal endophytes in *C. sativus* at different cultivation sites.

OTU	Taxon	Sampling Site			
		Bevagna	Moiano	S. Martino in Colle	Giano dell'Umbria
1	<i>Cadophora luteo-olivacea</i>	11	4		6
2	<i>Cadophora malorum</i>	4	2		2
3	<i>Botrytis cinerea</i>		1		
4	<i>Alternaria alternata</i>	9	2	1	4
5	<i>Alternaria infectoria</i>		1		
6	<i>Stemphylium vesicarium</i>		1	2	
8	<i>Epicoccum</i> sp.		6		
9	<i>Stagonosporopsis cucurbitacearum</i>			1	
10	<i>Spegazzinia</i> sp.		1		
11	<i>Aureobasidium pullulans</i>				3
12	<i>Aureobasidium pullulans</i>		1		
13	<i>Cladosporium cladosporioides</i>		1		
14	<i>Talaromyces pinophilus</i>	2	3	3	
15	<i>Talaromyces cecidicola</i>		4		
16	<i>Talaromyces assiutensis</i>			2	
17	<i>Aspergillus niger</i>	2			
18	<i>Aspergillus</i> sp.			1	
19	<i>Aspergillus flavipes</i>	1			
20	<i>Aspergillus europaeus</i>		1		
21	<i>Penicillium citrinum</i>		1		
22	<i>Fusarium oxysporum</i>	1	1		
23	<i>Ilyonectria</i> sp.			1	
24	<i>Parengyodontium album</i>	1			
26	<i>Ovatospora brasiliensis</i>			1	
27	<i>Malbranchea circinata</i>		1		
28	<i>Meyerozyma caribbica</i>		2		
29	<i>Rhodotorula</i> sp.	1	1	1	
30	<i>Coprinellus micaceus</i>		1		
31	<i>Peniophora</i> sp.		1		
32	<i>Filobasidium wieringae</i>				1
33	<i>Mucor fragilis</i>	5			
	Total No. isolates	37	36	13	16
	Species richness	10	20	9	5

Both Jaccard and Sorensen distance indices (Table S1c) showed divergences among sites, although these were not statistically significant (PERMANOVA $F = 1.1821$, $p = 0.2038$). Cluster analysis based on Jaccard distance showed the highest divergence occurring between S. Martino in Colle and Giano dell'Umbria and the lowest occurring between Bevagna and Moiano (Figure 3c). Clustering using Sorensen's dissimilarity gave us similar results (data not shown). PCoA analysis did not evidence clear groupings for both life stages and sites (Figure S2).

All the identified fungal genera were assigned to functional guilds (Table S2); most genera with a saprotroph trophic mode with respect to pathogens and symbionts were observed. The genus *Cadophora*, dominant in the corms, belongs to the "endophyte" guild and dark septate endophytes (DSE) living in plant roots [38].

3.3. Isolated Fungi from Rotten Plants

To isolate potential pathogenic fungi, some saffron plants visibly and widely colonized by molds were collected at the site of Moiano. In this case, 30 strains corresponding to eight OTUs were isolated from corms, stems, and leaves (Table 2) without performing the external disinfection treatment. Five OTUs belonged to Ascomycota, of which the order was: Hypocreales (two OTUs), Dothideales (one OTU), Pleosporales (one OTU), and Eurotiales (one OTU). Three OTUs belonged to Mucoromycota, order Mucorales. Most of the isolates (20) were obtained from corms, and, among these, the most frequent were *Fusarium oxysporum* (OTU22, nine of the 20 isolates) and *Rhizopus oryzae* (OTUs 38 and 39, eight of the 20 isolates). These latter OTUs were detected in stems and leaves too (Table 2).

Besides these two species and *Mucor circinelloides* (OTU37), which have been reported as plant pathogens [39–41], *Epicoccum nigrum* (OTU36), *Aureobasidium pullulans* (OTU11),

Trichoderma sp. (OTU35), and *Talaromyces pinophilus* (OTU14), known as endophytic fungi often involved in plant protection against phytopathogens [42–44], were found in rotten plants. Some of these endophytic species (OTUs 11 and 14) were detected in healthy plants too (Table 2).

4. Discussion

The endophytic community harboured by plants is among the key factors influencing the plant growth and health. In this study, the endophytic fungal communities associated with different tissues (corms, stems, leaves, tepals, and stigmas) of the medicinal and aromatic species *Crocus sativus* were examined and compared across ten Italian cultivation sites and in the different stages of the saffron life cycle. To isolate pathogenic fungal strains specific to *C. sativus* for future assays of endophyte–pathogen interaction, we also isolated mycelia from different tissues of some rotten saffron plants. We found 39 OTUs, and many of them were reported as endophytic species of saffron for the first time.

4.1. Biodiversity of Saffron Fungal Endophytes

A total of 135 strains from the endophytic community of *C. sativus* healthy plants were isolated in this work. Among these isolates, 34 OTUs were identified. The community was dominated by Ascomycota. This is consistent with previous results obtained from the same plant species [18,21] and more generally with findings that Ascomycota is the dominant group of endophytic fungi in many plant species from various environments [5]. Many of the fungi isolated in this study (21 OTUs) were identified as saffron endophytes for the first time, whereas they were previously reported as endophytic of other plant species (Table S3). Interestingly, some species here identified are not commonly reported as endophytic, e.g., *Hypoxylon fuscum* (OTU25) and *Coprinellus micaceus* (OTU30) are typically saprotrophic fungi associated with wood [45], whereas we found them in the leaves. It is worth mentioning that such species or species of the same genus have also been reported as endophytic in some plants (Table S3) [46–51], pointing to their putatively dynamic trophic modes (see also Section 4.3). Similarly, *Parengyodontium album* has been mainly reported as an environmental saprobe isolated from marine sediments [52], mineral materials in indoor environments [53], as well as from human infections [54]. Who et al. [55] reported this species as an endophyte of *Panax ginseng*, and we also isolated it from saffron corms. Moreover, to the best of our knowledge, some fungi isolated here, namely, *Talaromyces cecidicola*, and *Aspergillus europaeus*, have never been found as endophytic fungi in any other plant species. The presence of these fungi has been reported in different environments. *T. cecidicola* has been isolated from wasp insect galls [56], but we found it in saffron corms and stems. *A. europaeus* has been recently described as a novel species [57] and is known as a soil-borne fungus, but we isolated it from saffron stems. We cannot rule out the hypothesis of a contamination by air borne spores from non-endophytic fungi, during the isolation procedure; however, the endophytic status, as well as the plant protection and growth-promoting activities of these species, merit further investigations.

The saffron endophytic community was studied previously [17–21]. In the first and fourth studies, a few endophytic fungi were identified, and high antimicrobial activities were observed for some of them (*Penicillium*, *Alternaria*, and *Rhizopus oryzae*). Wani and colleagues [18] extensively investigated endophytic fungi and their properties in corms of plants cultivated in the Indian states of Jammu and Kashmir. These authors identified 36 OTUs. Among these, only three were also identified in the corms examined in this study, i.e., *Cadophora malorum*, *Talaromyces pinophilus*, and *Fusarium oxysporum*. Some other OTUs found by Wani and colleagues occurred in the other tissues examined: *Alternaria alternata* (in stems, leaves, tepals, and stigmas), *Botrytis cinerea* (leaves), *Epicoccum nigrum* (leaves), and *Aspergillus flavipes* (stigmas). The dominant species isolated from the corm tissue differed between this study and that of Wani et al. [18]: we observed the dominance of *Cadophora luteo-olivacea*, followed by *Cadophora malorum* and *Talaromyces pinophilus*, whereas Wani and colleagues found *Phialophora mustea* as the dominant species followed by

Cadophora malorum and *Talaromyces cellulolyticus*. *Cadophora* is a *Phialophora*-like anamorph genus, and the mentioned species of these genera share the same trophic mode in that they are all dark septate endophytes (DSE, Table S2; [58–60]. Therefore, the corm association with DSE likely holds an important ecological significance for *C. sativus*. DSE forms melanized septate hyphae and microsclerotia in the plant roots [61,62]. Fungal melanin likely gives structural rigidity to the cell wall and may support the fungal tolerance to abiotic stresses [63–65]. Additionally, DSE promotes plant growth by fostering the uptake of C, N, and P [66,67]. Interestingly, although Italian and Indian corms were both dominated by DSE endophytes, the most abundant species are different. Such different associations might have developed in the different cultivation areas and transmitted vertically since the host plant is propagated only vegetatively using corms. The different associations might be due either to different pre-existing fungal communities in these areas or to selective pressure because of different environmental conditions. Conversely, *Cadophora malorum* is the second dominant endophyte in both communities, suggesting a certain host specificity for this fungal species. Interestingly, in the study by Jan and colleagues [21], DSE were not found at all in the different tissues examined, and the dominant species were *Aspergillus ustus* and *Talaromyces pinophilus*. Moreover, Basidiomycete species were not found by these authors, and among Ascomycetes, only eight genera were detected. Ambardar and colleagues [19] adopted a different, culture-independent, metagenomic approach to detect fungal diversity associated with corms and roots of *C. sativus* cultivated in Kashmir (India), at the dormant and flowering stages. Interestingly, they found a still different situation, with a dominance of Zygomycota and Basidiomycota in the corms at the dormant and flowering stages, respectively.

Overall, different endophytic community structures were found in corms collected in the Italian and Indian areas. Here, diversity was detected even at a smaller geographical scale such as among near (7- to 46-km distance) cultivation sites within the Umbria region in Italy. Although differences were not statistically significant, we observed that some dominant species did not occur in some sites: *C. luteo-olivacea* and *C. malorum* were not detected at the site of S. Martino in Colle, and *T. pinophilus* was not detected in Giano dell'Umbria.

The endophytic fungal community of corms resulted significantly different among the different life stages: vegetative, dormant, and flowering. A lower diversity was observed in the dormant stage (where only DSEs occurred) with regard to the other stages. This result was somewhat expected since the spread of parasites is inhibited during the hot and dry Mediterranean summer, and saffron plants must enrich their endophytic community to cope with parasites in the hot (above 10–12 °C) and humid conditions that characterize soils in spring and autumn [14]. Ambardar et al. [19] also found stage-specific fungal associations in the corms at the dormant and flowering stages and higher levels of diversity at the flowering stage.

Finally, it is worth mentioning that the analysis of beta-diversity showed differences among saffron tissues, with the exception of flower tissues (stigmas and tepals), which were quite similar among each other. Many isolated taxa showed a preference for certain tissues. For example, *Stemphylium vesicarium* and *Epicoccum* spp. occurred in leaves only, whereas *Mucor fragilis* and *Aureobasidium pullulans* occurred in flower organs only; additionally, corms shared few endophytic species with other tissues (Table 2). Such tissue-specificity has also been observed in the study by Ambardar et al. [19], as well as in different plant species [16,34,68], and it might be related to the ability of endophytes to use specific substrates. Moreover, this result is expected considering the different types of ecological challenges, such as phytopathogens and other biotic/abiotic stresses, that the different plant organs must cope with.

4.2. Potential Biotechnological Importance of Saffron Endophytes

Endophytic fungi are known as a major source of novel bioactive metabolites useful in several applications, including agriculture, human therapy, and industrial biotech-

nology. The most important bioactive components produced by *Crocus sativus* are the apocarotenoids crocetin, crocin, safranal, and picrocrocin [69], and the involvement of endophytic fungi in the biosynthesis of such molecules has been reported [70]. Several endophytic fungi identified in this study are known for certain bioactivities. For example, among the three *Talaromyces* spp. detected in corms, stems, and leaves, *T. pinophilus* is an interesting species since it can inhibit fungal phytopathogenic species [44] and holds interesting biotechnological potential because of its useful reservoir of biomass-degrading enzymes, such as α -amylase, cellulase, endoglucanase, etc. [71], and secondary metabolites with insecticidal activity [72]. Similarly, *T. assiutensis* has been previously reported as an efficient nematocidal species [73] in olive nurseries and a valuable source of antimicrobial metabolites [74]. Its occurrence in saffron may be linked to these properties, as nematodes are among the worst saffron enemies [75]. The nematocidal activity was also previously observed for *Fusarium oxysporum* [76]. We detected this species in corms of both healthy and rotten plants (see the next section).

Considering tissues other than corm, *Alternaria alternata* and *Epicoccum nigrum* were among the dominant taxa (Table 2). According to Wani et al. [18] *A. alternata* has interesting antimycotic potentials, whereas the *Epicoccum* genus is known for the production of diverse classes of biologically active secondary metabolites holding cytotoxic, anticancer, antimicrobial, and anti-diabetic activities [77].

4.3. Plant Growth-Promoting, Pathogenic, and Anti-Pathogenic Effects of Saffron-Associated Fungi

The term endophytes is generally used to describe microorganisms (fungi and bacteria) that live within the tissues of healthy plants. However, defining a fungal species as endophytic is controversial, as, according to Schulz and Boyle [78], the endophytic condition must be considered a momentary status because plant–endophyte interaction may change in time depending on several factors. In some cases, the plant–endophyte relationship may be mutualistic as fungi can benefit from the environment and nutrients provided by the host. In turn, the endophyte may produce plant growth-promoting molecules, such as auxin, compounds with anti-fungal or anti-bacterial properties or properties toxic to insect pests or grazing animals [23,79,80]. Endophytes can also enhance resistance to pathogens by triggering host defense mechanisms [81]. For several fungal endophytes, latent pathogenicity has been documented. The transition from a symptomless, endophytic condition to a pathogenic stage may depend on physiological changes in the host, such as abiotic stress, growing stages, and interaction with other microorganisms [82]. Additionally, virulence genes can be activated or deactivated by mutations [83]. Moreover, the colonization of different host species and plant organs can cause a fungus to adopt contrasting lifestyles [84,85]. In agreement with this, some of the most abundant endophytes identified as DSE in this study belong to the genus *Cadophora*, which is also known as phytopathogenic in different plant species: *C. luteo-olivacea* is the causal agent of post-harvest diseases of kiwifruit, grape, and pears [86–88]; similarly, *C. malorum* is a postharvest pathogen on apples and pears, and it can attack *Asparagus* spp. [89] and cause wood discoloration and decay on the trunks of old kiwifruit “Hayward” vines [90]. Thus, these fungi have likely alternative DSE/pathogen trophic modes. In some cases, plant growth-promoting activity was also documented, i.e., for *C. luteo-olivacea* in *Allium porrum* [91] and for *C. malorum* in saffron [18]. According to this last finding, we did not find *Cadophora* strains in the rotten saffron plants. Moreover, several fungal species were detected in both healthy and rotten plants, suggesting a condition of latent pathogenicity for these endophytes. *Talaromyces pinophilus*, abundantly found in healthy corms and occurring in stems (Table 2), has been shown to be a low-risk or opportunistic pathogen in *C. sativus*, [18] and, interestingly, we found it in rotten corms too.

The corm rot caused by *F. oxysporum* has been reported as the worst disease for saffron, causing severe losses in many saffron fields [39]. Wani and colleagues [70] reported that the *C. sativus* endophytic fungus *Mortierella alpina* enhances the plant tolerance against this pathogen. We detected *F. oxysporum* at high frequency in the rotten plants, especially

in the corms. Interestingly, a *F. oxysporum* strain was also isolated from the corm of a healthy plant collected in the same site of rotten plants (Moiano), suggesting a likely changing lifestyle of *F. oxysporum* in saffron corms. In this regard, Wani et al. [18] showed *F. oxysporum* to behave as a latent pathogen in saffron, with high plant growth-promoting and antimycotic potential. In agreement with this, the pathotroph as well as the saprotroph and symbiotroph trophic modes were documented for the genus *Fusarium* (Table S2). All the *F. oxysporum* strains isolated in this study shared the same ITS sequence as well as that of Wani et al. [18] (data not shown). It would be interesting to perform further analysis to evaluate if the isolates colonizing healthy and rotten plants are genetically different or if they represent the same *F. oxysporum* strain.

Epicoccum nigrum and *Alternaria alternata* were reported as latent pathogens in saffron [18]. We isolated an *E. nigrum* strain from the stem of the rotten plant that differed for the ITS sequence from the *Epicoccum* strains isolated from leaves of healthy plants collected in the same cultivation site of Moiano. Interestingly, the *E. nigrum* strain we isolated from rotten plants shared the same ITS with the strain found in corms by Wani et al. [18]. *A. alternata* was abundantly found in aerial tissues of healthy plants examined in this study but not in rotten plants.

Stemphylium vesicarium can cause diseases in many crops and non-crop species including garlic, onion, asparagus, and pear [92], and it was here isolated for the first time in saffron healthy leaves, suggesting possible latent pathogenicity. According to FUNGuild, assignment *Stemphylium* spp. can behave both as pathogenically and saprotrophically. Another species abundantly found in rotten plants (corms, stems, and leaves) is *Rhizopus oryzae*. To the best of our knowledge, this species has not been previously reported as a saffron pathogen; rather, it has been found only in healthy saffron roots of Moroccan cultivation, with high antibacterial and antioxidant activities [20]. However, *R. oryzae* is a potent pathogen of several crop species [40,93,94], and its latent pathogenicity in saffron should be investigated. Another fungus we found in rotten plants was *Mucor circinelloides*; it is known as a phytopathogen [41]; however, it was never reported as a saffron-associated species. *Trichoderma* and *Aureobasidium* spp., here isolated from rotten plants, are well-known taxa for their plant protective activities [43,95]; thus, their potential role as pathogen antagonists needs to be further investigated.

5. Conclusions

This study provides novel insights concerning the biodiversity of the fungal endophytic communities inhabiting both aerial and below-ground parts of saffron plants, cultivated in different Italian sites. Tepals and stems were examined for the first time for the endophytic biodiversity. Saffron-specific fungal pathogens from rotten plants were also isolated. Most of the endophytes were found for the first time in *Crocus sativus* with respect to previous studies, and some species were newly identified as endophytic. All fungal strains were long-term conserved ex-situ and will be used in future endophyte–pathogen interaction assays, to evaluate the potential of endophytic strains in the biological control of plant fungal pathogens. In addition, the fungal strain collection is available as a resource for the identification of bioactive molecules of agronomical and biotechnological interest.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/d13110535/s1>, Figure S1: Map of the study sites and pairwise distances among them. Figure S2: PCoA plot based on Jaccard distances. Table S1a: Dissimilarity values between tissues based on Jaccard and Sorensen indices. Table S1b: Dissimilarity values between life stages based on Jaccard and Sorensen indices. Table S1c: Dissimilarity values between collecting sites based on Jaccard and Sorensen indices. Table S2: Assignment of the identified fungal genera to functional guilds by FUNGuild annotation title, Table S3: Occurrence in other plant species of the endophytic OTUs identified in *C. sativus* for the first time.

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