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Exploring Endophytic Fungi from *Humulus lupulus* L. for Biocontrol of Phytopathogenic Fungi

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Abstract: *Humulus lupulus* L. (hop) is a crucial crop within the brewing industry and a rich source of bioactive compounds. Traditionally concentrated in northeast regions of Europe, hop cultivation has expanded towards southern territories such as Italy over recent decades. Managing phytosanitary threats in Mediterranean climates poses challenges due to limited knowledge and registered agrochemicals. In pursuit of eco-friendly alternatives for disease management, we isolated 262 endophytic fungal strains from wild hop roots, stems, leaves, and flowers. Through phylogenetic analyses, we identified 51 operational taxonomic units. Dominant species such as *Ilyonectria macrodidyma*, *Penicillium* sp., *Diaporthe columnaris*, *Plectosphaerella cucumerina*, and *Fusarium oxysporum* were exclusive to roots. In contrast, *Alternaria* spp. and *Epicoccum* spp. were prevalent in other tissues, and *Botrytis cinerea* was exclusively detected in female flowers. We tested seven isolates—*Epicoccum* sp., *Aureobasidium pullulans*, *Plectosphaerella cucumerina*, *Stemphylium vesicarium*, *Periconia byssoides*, *Talaromyces wortmannii*, and *Nigrospora sphaerica*—against the four phytopathogenic fungi *Alternaria* sp., *Fusarium oxysporum*, *Botrytis cinerea*, and *Sclerotinia sclerotiorum*. All endophytes exhibited antagonistic effects against at least one pathogen, with *Plectosphaerella cucumerina* showing the strongest inhibition against *Alternaria* sp. This study marks the first exploration of endophytic fungi from various hop tissues. All isolated strains were ex situ conserved for future bioactivity assessments and biotechnological applications. Original data with a key relevance for the environmentally friendly management of plant diseases are provided.

Keywords: hop cultivation; biological control; internal transcribed spacer; pathogenic fungi; *Alternaria* sp.; *Plectosphaerella cucumerina*

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1. Introduction

Hop (*Humulus lupulus* L.) is a dioecious, perennial, and herbaceous climbing plant of the Cannabaceae family. While it naturally thrives in temperate areas, it is also cultivated for its secondary metabolites, which impart flavor, bitterness, aroma, and antimicrobial properties to beer. Moreover, certain metabolites exhibit bioactive properties with pharmaceutical potential, notably as sedatives and antimicrobial agents [1]. Hop has also garnered attention for its possible cancer chemopreventive effects [2,3]. Over 1000 chemicals have been identified in hop, primarily comprising essential oils, α- and β-acids, and prenylflavonoids, which accumulate in the resinous substance (lupulin) of the female flowers, called cones [4]. According to the 2021 FAO report, global annual hop production and harvesting areas increased by 34% and 18%, respectively, from 2011 to 2021. The United

States of America and Germany stand out as the most prolific hop-producing countries. However, the European Union remains the primary contributor to global hop production, accounting for nearly 50% of the total output. While hop cultivation in Europe is traditionally concentrated in the northeast regions like Germany, the Czech Republic, and southeast England, in the last decades, the cultivation borders have moved towards southern European countries, including Italy. Despite the natural growth of hop plants across the entire peninsula, Italy relies heavily on imports, with 98% of its hop requirements are sourced from external suppliers [5]. In recent years, the growing interest in hop cultivation stems from the proliferation of microbreweries producing craft beers with diverse tastes and flavors. These breweries are increasingly inclined to use locally cultivated hops rather than relying on imports (https://www.assobirra.it/wp-content/uploads/2021/06/AssoBirra_AnnualReport_2020_giugno2021_DEF.pdf, accessed on 20 January 2024). Moreover, the Italy's remarkable variety of pedoclimatic characteristics could influence and shape hop organoleptic qualities. However, there is a notable lack of experience and knowledge in Italy regarding hop cultivation practices, phenology, and yard management, hindering the development of this burgeoning sector. Mediterranean climatic conditions present particular challenges, including the threat of phytopathogenic fungi, insect pests, mites, viruses, and viroids, all of which pose significant risks to hop production in terms of both yield and quality [6,7]. Concerning pathogenic fungi, the most prevalent hop diseases include downy mildew caused by *Pseudoperonospora humuli* and powdery mildew caused by *Podosphaera macularis*, alongside various types of rots, wilts, and others. Current management strategies primarily involve the use of resistant cultivars and fungicides targeting *P. macularis* [6,8]. However, the use of fungicides and other plant protection products against the different pests is challenged by the lack of registered active substances for hops under Italian legislation [7].

Plant endophytic fungi colonize the intercellular spaces of living plant tissues without triggering disease symptoms [9]. The inner part of the plant is a protected niche containing the necessary nutrients for fungal survival and growth. Colonization can occur in tissues of one or more parts of the host plant, including roots, stems, leaves, reproductive systems, and fruits. In exchange for this safe place, endophytic fungi may improve plant fitness by different mechanisms ranging from biological control of phytopathogens to biofertilization and stress tolerance. Such benefits can occur directly and/or directly. The direct mechanisms include the increase in plant nutrient acquisition and phytohormones production, which are directly related to the increase in biomass, root system expansion, plant height, and weight. Tolerance to biotic and abiotic stresses as well as activation of systemic resistance and production of antibiotics and secondary metabolites are considered indirect aspects of such growth promotion [10]. Exploring plant–endophyte interactions across various crops is pivotal for fostering sustainable cultivation practices. Many endophytic species produce antibiotics and antifungal compounds that safeguard plants against pathogens, offering promising avenues for eco-friendly and economically sustainable agriculture [11]. The advantages of using fungi in agriculture include greater biosafety and less environmental and human health risk, specificity for the target pest and others. Indeed, the utilization of endophytic fungi in agriculture for biological control of phytopathogens has garnered increasing attention during the last decades [12], driven by the increasing demand for sustainable and environmentally friendly alternatives to chemical products [13]. However, research on endophytic microorganisms for disease control in hops remains limited [14].

The aim of this study was to isolate endophytic fungi from hop plants, characterize them taxonomically, and test their bioactivity against agriculturally significant phytopathogenic fungi. To achieve this, an ex situ collection of endophytic fungal strains was established by isolating fungi from various tissues (roots, stems, leaves, and both female

and male flowers) of wild *H. lupulus* accessions collected from different sites in Central Italy. To the best of our knowledge, this research presents the initial comprehensive report on the endophytic fungi associated with *H. lupulus* and their potential application in agriculture as biocontrol agents against phytopathogenic fungi.

2. Materials and Methods

2.1. Biological Material and Study Sites

Wild, healthy plants of *H. lupulus* were collected between 2017 and 2019 from ten natural sites in Central Italy: seven in the Umbria region, two in the Marche region, and one in the Lazio region (Table 1). A total of 36 plants were collected, ranging from 1 to 6 per site. Different tissues, namely roots, stems, leaves, female flowers (cones), and male flowers, were collected. Due to factors such as harvesting time/life stage, sex, and accessibility to various plants parts, not all tissues could be collected from every plant or site. However, cones and leaves were obtained from nearly all plants and sites (Table 1).

Table 1. Sampling sites and number of isolates from the different sites and tissues.

Sampling Site	Locality	Region	Latitude	Longitude	No. Isolates per Tissue					
					Roots	Stems	Leaves	Cones	Male Flowers	Total
1	Ponte San Giovanni	Umbria	43.092056	12.461544		4	22	5		31
2	Valfabbrica	Umbria	43.327520	12.718487			12	11		23
3	Città di Castello	Umbria	43.457615	12.230473	10	6	16	23	7	62
4	Belfiore	Umbria	42.981651	12.734363	5	5	10	14		34
5	Nera River	Umbria	42.734921	12.833146			4	19		23
6	Piediluco	Umbria	42.530727	12.733482	12	9	25	5		51
7	Orvieto	Umbria	42.697959	12.211644			2	3		5
8	Bolsena Lake	Lazio	42.638573	11.889101			7	3	4	14
9	Potenza River	Marche	43.246611	13.219093				5		5
10	Penna San Giovanni	Marche	43.049822	13.464626				14		14
			Total no. isolates		27	24	98	102	11	262

2.2. Isolation of Fungi and Molecular Identification

All plant tissues were surface-sterilized basically as described by Belfiori and colleagues [15]: They were treated with 0.3% sodium hypochlorite for 3 min, 70% ethanol for 1 min, and then rinsed three times with sterile distilled water. As a control of the sterilization, the last water rinse was incubated in PDA to exclude any fungal growth. Tissues were air-dried under sterile conditions, cut into small segments (0.5–1 cm) using a sterile surgical blade, and placed on potato dextrose agar (PDA, Merck) supplemented with 100 mM ampicillin to prevent bacterial contamination. The cultures were then incubated at 25 °C and inspected every 3–4 days for the emergence of hyphae from the tissues, up to approximately 4 weeks. For each tissue sample and each sampling site, all mycelia from colonies exhibiting different morphologies were picked and re-inoculated onto fresh PDA in Petri dishes to obtain pure cultures. Finally, the single cultures were transferred into potato dextrose broth (PDB, Merck) with 50% (*v*/*v*) glycerol, frozen in liquid nitrogen, and kept at −70 °C for long-term storage. The obtained fungal isolates are deposited in the collection of the BioMemory Project (https://biomemory.cnr.it/collections/CNR-IBBR-FABI, accessed on 20 January 2024).

2.3. OTUs Molecular Identification and Phylogenetic Analysis

Genomic DNA was isolated from each strain as described in Arnold and Lutzoni [16]. A small amount (0.3 g) of mycelium was ground and resuspended 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 at 14,000 rpm for 10 min. The supernatant was precipitated in an equal volume of isopropanol for 30 min at −20 °C. The DNA was pelleted by maximum-speed

centrifugation for 20 min at 4 °C, vacuum-dried, and resuspended in 50 µL of double-distilled nuclease-free water. DNA concentration was measured using a NanoDrop 2000 UV–vis Spectrophotometer (Thermo Scientific, Waltham, MA, USA). The ITS region was amplified by PCR with the primers ITS1f [17] and ITS4 [18]. PCR was carried out in a 25 µL reaction mixture containing template DNA (10 ng), 10X reaction buffer (RBC Bioscience, New Taipei City, Taiwan), 4 mM $MgCl_2$, dNTPs (0.2 mM each), 10 µM of each primer, and 1 U of RBC Taq polymerase (RBC Bioscience). A GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA) was used 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 performed using the primers ITS1f, ITS4, 5.8sf, and 5.8sb [18,19] 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). Assembly, editing, and alignment of sequences were conducted using GENEIOUS version 4.8.5. The resulting DNA sequences were deposited in GenBank, under accession numbers OQ257045 to OQ257306. Similarity searches were performed in GenBank database using BLASTn [20]. In order to designate operational taxonomic units (OTUs), sequences were clustered using a 97% similarity threshold using CD-HIT-EST [21] (https://github.com/weizhongli/cdhit, accessed on 27 January 2025). The functional diversity of the identified fungal genera was analyzed according to the database FungalTraits [22]. Phylogenetic analyses were performed to better identify the OTUs in the Sordariomycetes and Dothideomycetes classes that were the most prevalent. To this purpose, based on BLASTn searches, similar sequences were downloaded from GenBank and aligned with the sequenced OTUs. Multiple sequences alignments were performed using MAFFT version 7 [23] with the L-INS-I parameters. Trees were generated using RaxML version 8.2.12 software [24] using the following options: rapid bootstrapping with auto MRE, GTRGAMMA distribution model, and empirical base frequency. The species names of sequences downloaded from GenBank are reported in the trees along with the accession numbers. Alignments and trees were deposited in TreeBASE under accession no. 31340.

2.4. Diversity Analyses

Species richness was calculated for each tissue as the number of different OTUs. Since different numbers of roots, stems, leaves, and flowers were sampled and from different plants, we could not calculate the relative abundance of each OTU per plant. However, the relative abundance of each OTU was calculated for each tissue by dividing the number of isolates of an OTU in that tissue by the total number of isolates from that tissue. The dominant species were determined for each tissue according to Rivera-Orduña et al. [25] as the OTUs with Pi > 1/S, with Pi being the relative abundance and S the species richness.

2.5. Antifungal Activity of Isolated Endophytic Fungi

Among the isolated endophytes, seven strains (see results) belonging to various phylogenetic groups and with a different tissue distribution were selected for testing. Four pathogenic species were used for these tests: *Botrytis cinerea* (AR593) and *Sclerotinia sclerotiorum* (AR397) were previously isolated from the basal part of the petiole of symptomatic leaves of *Vitis vinifera*, whereas *Alternaria* sp. (strain E140) and *Fusarium oxysporum* (strain E529) were isolated from asymptomatic hop tissues. However, their pathogenicity was demonstrated in experiments on tobacco leaves (unpublished results).

2.5.1. Dual-Culture Assays

Endophytic fungi were tested against pathogenic fungi by dual-culture assays. Briefly, 5 mm diameter mycelial plugs from the edge of young cultures of both the endophytic and pathogen fungus were placed on the opposite sides of a 9 cm diameter Petri plate

containing PDA at about 1 cm from the margin. Control plates were prepared by growing the endophytic and the pathogenic strains in single cultures in the same conditions. Three replicates were made for each treatment. Following an incubation period of approximately seven days at 25 °C, plates were inspected, and the presence of inhibition signals in the interaction zone between the two mycelia was assessed to determine bioactivity.

2.5.2. Agar Diffusion Method

We followed the procedure described by Hajieghrari et al. [26] with some modifications. The endophytic fungi to be tested were grown on PDA plates for about seven days. Subsequently, a mycelial agar disc (5 mm in diameter) was excised from the culture's periphery and inoculated in 100 mL of sterile PDB in a 250 mL conical flask. The flasks were incubated at 25 °C on a rotary shaker at 100 rpm for 14 days. The culture was filtered with one or two layers of Miracloth (Millipore, Burlington, MA, USA) to remove mycelial parts and then sterilized using 0.2 µm pore filters (Minisart® Syringe Filters, Sartorius, Goettingen, Germany). The filtrate was added to PDA medium molten at 43 °C at a final concentration of 20% (v/v) and poured into Petri dishes. A 5 mm diameter plug of pathogenic mycelium was put on the center of the PDA plates containing the endophytic extract and incubated at 25 °C for 6–8 days. Control plates were prepared for growing the pathogenic fungus in PDA containing PDB 20% v/v. Three replicates were performed for each treatment. Radial growths of the mycelia were recorded at 6 to 8 days of incubation, and the percentage of inhibition of the pathogen growth was calculated according to Edington et al. [27] as follows:

$$I = [(C - T)/C] \times 100$$

where I indicates the percentage of inhibition, C the radial growth of the pathogen in control plates, and T the radial growth of the pathogen in the presence of the endophytic broth. The percentage of inhibition was calculated for each treatment as an average among the three replicates.

3. Results

3.1. Isolation and Identification of Hop Endophytic Fungi

A total of 262 fungal isolates were recovered from roots (27), stems (24), leaves (98), cones (102), and male flowers (11) of *H. lupulus*. The isolates were identified through analyses of the ITS ribosomal gene sequence. Clustering of the sequences at 97% of identity allowed the detection of 51 OTUs (Table 2). The putative species names, inferred by BLASTn searches and phylogenetic analysis (Figure 1a,b), are reported in Table 2. More specifically, 45 OTUs (88.2%) belonged to Ascomycota, 5 (9.8%) to Basidiomycota, and 1 (2%) to Mucoromycota. All the Ascomycota OTUs belonged to Pezizomycotina and clustered in four classes: Sordariomycetes and Dothideomycetes were the most represented (20 OTUs each), followed by Eurotiomycetes (4 OTUs) and Leotiomycetes (1 OTU). Among Ascomycota, the order with the highest number of OTUs was Pleosporales (16 OTUs), followed by Hypocreales and Diaporthales (6 OTUs each), Glomerellales (5 OTUs), Eurotiales (4 OTUs), Xylariales (3 OTUs), and Dothideales, Cladosporiales, Mycosphaerellales, Botryosphaeriales, and Helotiales (1 OTU each). All the Basidiomycota OTUs belonged to Agaricomycotina, in the classes of Tremellomycetes (three OTUs) and Agaricomycetes (two OTUs). Basidiomycota were represented by four orders, namely Tremellales (two OTUs), Polyporales, Cantharellales, and Cystofilobasidiales (one OTU each). Mucoromycota were represented by one OTU belonging to the class Mucoromycetes, order Mucorales.

Table 2. Description of the isolated OTUs, their distribution in the different tissues of *Humulus lupulus,* relative abundance, and dominant species.

Isolates			Closest Match in GenBank			No. of Isolates						Pi				
OTU	**Strain**	**Accession No.**	**Classification**	**Accession No.**	**Similarity (%)**	**Roots**	**Stems**	**Leaves**	**Cones**	**Male Flowers**	**Total**	**Roots**	**Stems**	**Leaves**	**Cones**	**Male Flowers**
			ASCOMYCOTA													
			DOTHIDEOMYCETES													
			Pleosporales													
1	E140	OQ257068	*Alternaria* sp.	AF347031	100		7	23	45	7	82		0.304 *	0.235 *	0.447 *	0.636 *
2	E333	OQ257172	*Alternaria* sp.	HG936477	96				1		1				0.010	
3	E332	OQ257171	*Alternaria* sp.	AF347031	94.8				1		1				0.010	
4	E150	OQ257077	*Epicoccum* sp.	HQ630972	100		5	24	9	2	40		0.174 *	0.245 *	0.087 *	0.182
5	E137	OQ257065	*Alternaria infectoria*	MK461061	99.69			2	2	1	5			0.020	0.019	0.091
6	E149	OQ257076	*Stemphylium vesicarium*	MK461018	99.68		2	2			4		0.087 *	0.020		
7	E302	OQ257143	*Bipolaris sorokiniana*	KU194490	100			3			3			0.031		
8	E354	OQ257187	*Periconia macrospinosa*	JX981482	99.8				2		2				0.019	
9	E358	OQ257191	*Pithomyces chartarum*	MH860227	100				1		1				0.010	
10	E573	OQ257304	*Neosetophoma italica*	KP711356	99.84		1				1		0.043			
11	E165	OQ257091	*Parastagonospora nodorum*	KX928830	98.89			1			1			0.010		
12	E343	OQ257177	*Periconia byssoides*	KC954157	99.8				1		1				0.010	
13	E307	OQ257148	*Pyrenophora tritici-repentis*	KT692571	99.6			1			1			0.010		
14	E194	OQ257100	*Neodidymelliopsis cannabis*	MH859057	99.08				1		1				0.010	
50	E546	OQ257285	*Paraphoma* sp.	DQ420980	91		1				1		0.043			
51	E375	OQ257207	*Sporormiella intermedia*	JX136249	99.10			1			1			0.010		
			Dothideales													
15	E157	OQ257083	*Aureobasidium pullulans*	FN868454	100		1	7	2		10		0.043	0.071	0.019	
			Cladosporiales													
16	E159	OQ257085	*Cladosporium* sp.	HQ631003	100			11	12	1	24			0.112 *	0.117 *	0.091
			Mycosphaerellales													
17	E559	OQ257293	*Mycosphaerella* sp.	EU167596	100			1			1			0.010		
			Botryosphaeriales													
18	E431	OQ257234	*Diplodia sapinea*	MF398866	98.1				1		1				0.010	
			SORDARIOMYCETES													
			Diaporthales													
19	E374	OQ257206	*Diaporthe novem*	MH864504	100	1	1	3	5		10	0.037	0.043	0.031	0.049 *	
20	E126	OQ257055	*Diaporthe oncostoma*	LN714541	99.83		1	4			5		0.043	0.041 *		
21	E367	OQ257200	*Diaporthe* sp.	KJ482538	100			3	1		4			0. 031	0.010	
22	E321	OQ257161	*Diaporthe foeniculina*	AY620999	100		1		2		3		0.043		0.019	
23	E518	OQ257262	*Diaporthe columnaris*	MN450640	99.8	2					2	0.074 *				
24	E494	OQ257241	*Cytospora* sp.	AY618229	98.4		1				1		0.043			

Table 2. *Cont.*

Isolates			Closest Match in GenBank			No. of Isolates						Pi				
OTU	**Strain**	**Accession No.**	**Classification**	**Accession No.**	**Similarity (%)**	**Roots**	**Stems**	**Leaves**	**Cones**	**Male Flowers**	**Total**	**Roots**	**Stems**	**Leaves**	**Cones**	**Male Flowers**
			Glomerellales													
25	E365	OQ257198	*Colletotrichum coccodes*	AJ301984	99.50			1	2		3			0.010	0.019	
26	E346	OQ257180	*Colletotrichum gloeosporioides*	AJ301907	100			1	1		2			0.010	0.010	
27	E545	OQ257284	*Plectosphaerella cucumerina*	KF472138	98.8	2					2	0.074 *				
28	E432	OQ257235	*Colletotrichum karsti*	MW081181	99.19			1			1			0.010		
29	E555	OQ257291	*Colletotrichum acutatum*	AJ301971	100		1				1		0.043			
			Hypocreales													
30	E569	OQ257300	*Ilyonectria macrodidyma*	JN859422	100	7					7	0.259 *				
31	E542	OQ257282	*Fusarium* sp.	MK408102	99.8	1	1	2	1		5	0.037	0.043	0.020	0.010	
32	E347	OQ257181	*Fusarium sambucinum*	KM231813	99.8	1	1	1	2		5	0.037	0.043	0.010	0.019	
33	E529	OQ257272	*Fusarium oxysporum*	MT453296	99.82	2		1			3	0.074 *		0.010		
34	E523	OQ257267	*Fusarium oxysporum*	AY928418	100	2					2	0.074 *				
35	E519	OQ257263	*Fusarium verticillioides*	KJ957786	99.8	1					1	0.037				
			Xylariales													
36	E331	OQ257170	*Nemania serpens*	KU141386	100				1		1				0.010	
37	E521	OQ257265	*Truncatella angustata*	MT514368	99.6	1					1	0.037				
38	E425	OQ257228	*Nigrospora sphaerica*	HQ608063	100				1		1				0.010	
			LEOTIOMYCETES													
			Helotiales													
39	E235	OQ257130	*Botrytis cinerea*	MH860108	100				8		8				0.078 *	
			EUROTIOMYCETES													
			Eurotiales													
40	E554	OQ257290	*Penicillium* sp.	KF367497	100	3					3	0.111 *				
41	E490	OQ257238	*Penicillium* sp.	MN861278	98.04	1					1	0.037				
42	E161	OQ257087	*Aspergillus* sp.	MK461022	99			1			1			0.010		
43	E536	OQ257278	*Talaromyces wortmannii*	NR_172039	99.8	1					1	0.037				
			BASIDIOMYCOTA													
			AGARICOMYCETES													
			Polyporales													
44	E148	OQ257075	*Hyphodermella rosae*	MF475983	99.84			1			1			0.010		
			Cantharellales													
45	E544	OQ257283	*Rhizoctonia solani*	MH862557	99.69	1					1	0.037				
			TREMELLOMYCETES													
			Cystofilobasidiales													
46	E557	OQ257292	*Itersonilia perplexans*	MH861890	99.69			1			1			0.010		

Table 2. *Cont.*

Isolates			Closest Match in GenBank			No. of Isolates						Pi				
OTU	**Strain**	**Accession No.**	**Classification**	**Accession No.**	**Similarity (%)**	**Roots**	**Stems**	**Leaves**	**Cones**	**Male Flowers**	**Total**	**Roots**	**Stems**	**Leaves**	**Cones**	**Male Flowers**
			Tremellales													
47	E142	OQ257070	*Vishniacozyma heimaeyensis*	KY105824	100			1			1			0.010		
48	E143	OQ257071	*Cryptococcus* sp.	EU852359	99.8			1			1			0.010		
			MUCOROMYCOTA													
			MUCOROMYCETES													
			Mucorales													
49	E520	OQ257264	*Mucor fragilis*	GU566275	99.8	1					1	0.037				
				Total no. isolates		27	24	98	102	11	262					
				Species richness		14	13	25	20	4	51					
				1/S								0.067	0.077	0.040	0.045	0.250

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

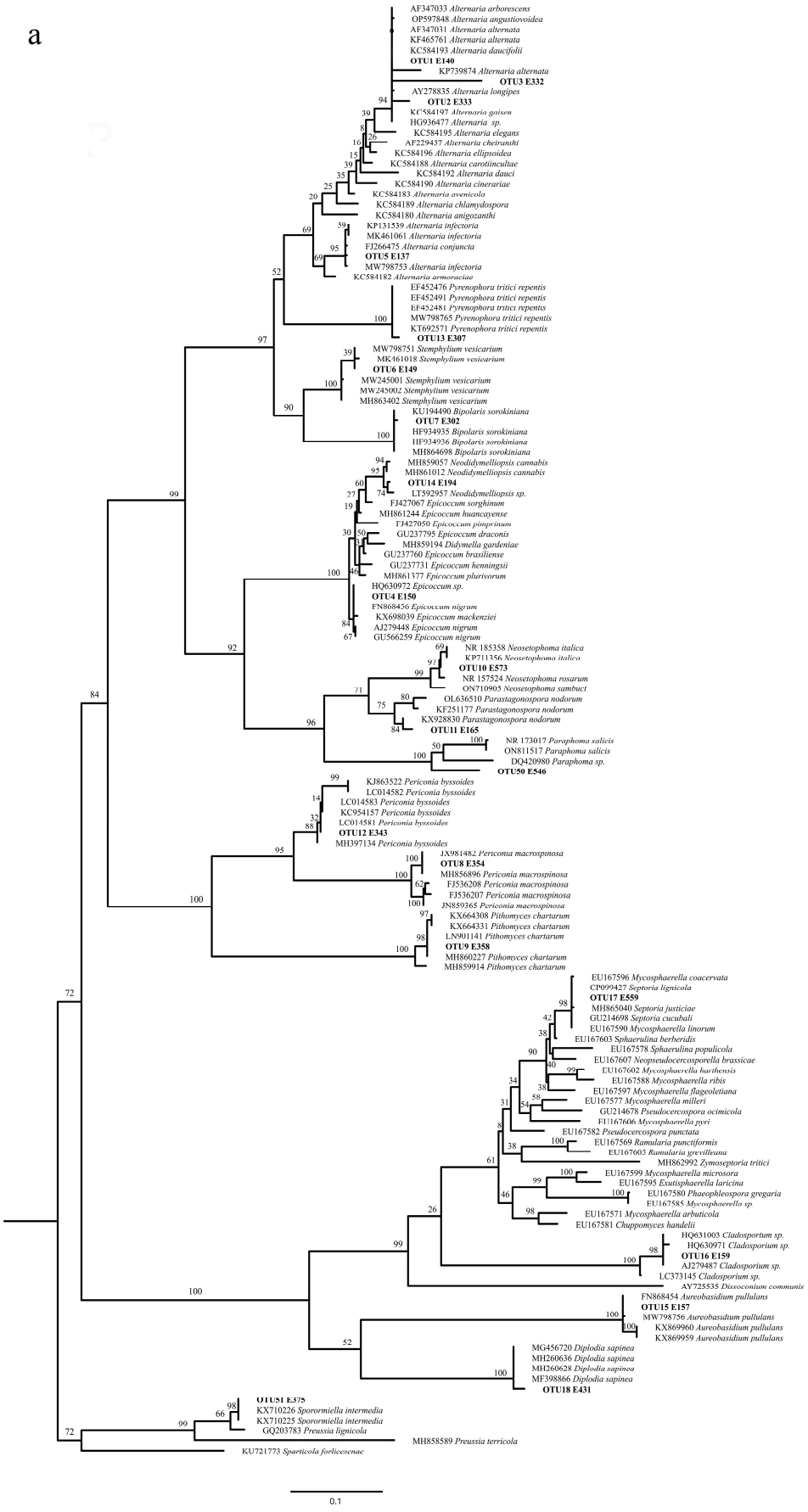

Figure 1. *Cont.*

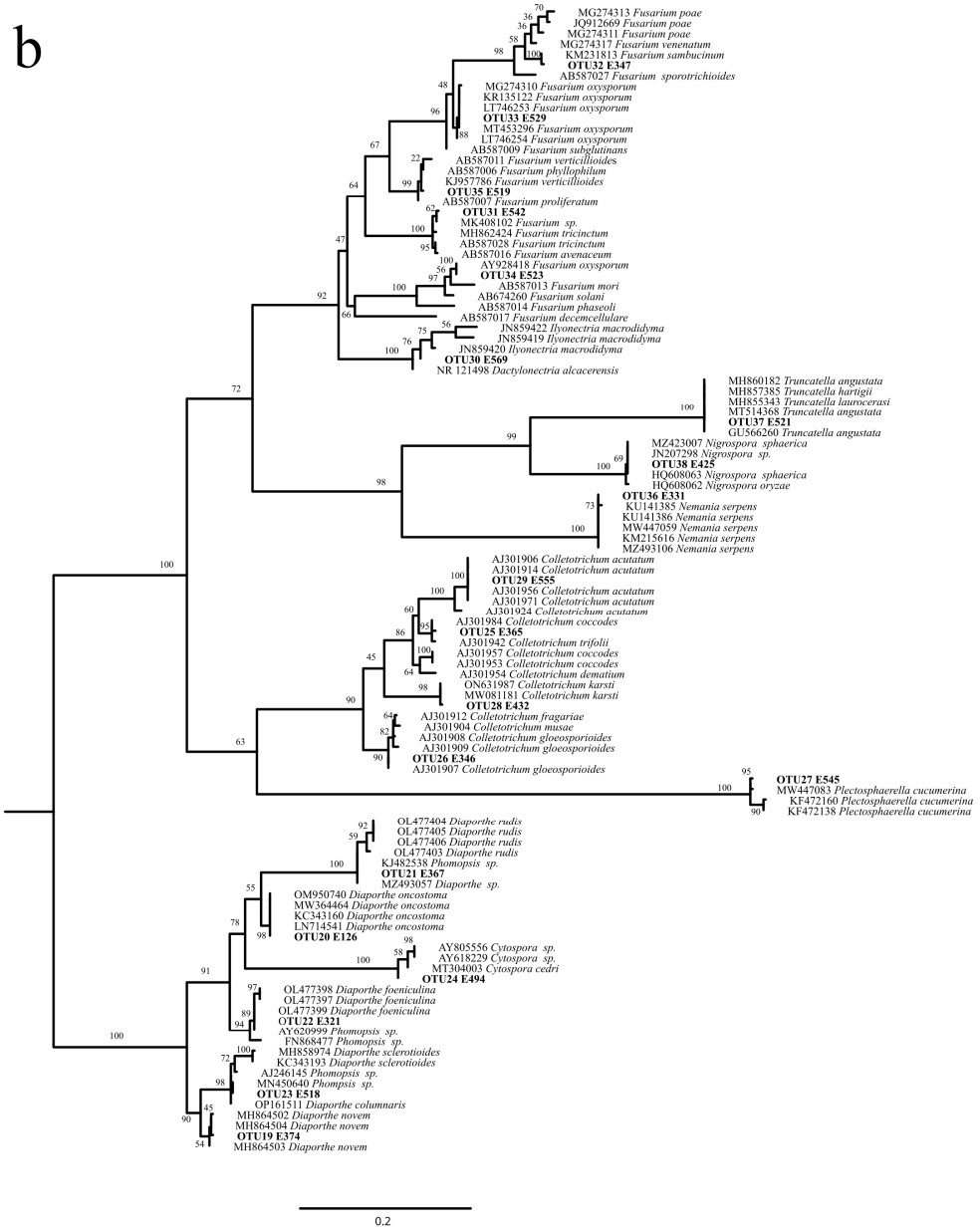

Figure 1. ITS based phylogenetic trees of Sordariomycetes (**a**) and Dothideomycetes (**b**), showing the position of the OTUs identified in this study (in bold). GenBank accession numbers of sequences downloaded from GenBank are reported near the taxa names. Numbers near the branches indicate bootstrap values (percentage over 1000 replicates).

3.2. Characterization and Distribution of Endophytic Fungi in the Different Tissues and Sampling Sites

Species names were assigned to 37 out of the 51 OTUs, whereas 14 OTUs were identified at the genus level only (Table 2). The alpha diversity, measured as species richness (S), ranged from 4 to 25 in the different plant tissues. Roots, stems, and male flowers had a higher S than leaves and cones, with respect to the number of isolates obtained from each tissue (Table 2). Thirteen OTUs were shared between different tissues; in particular, *Alternaria* sp., *Epicoccum* sp., *Diaporthe novem*, and *Fusarium* were the most common taxa among tissues (Table 2).

Dominant species were identified in each tissue as those species with Pi > 1/S (see Section 2 and Table 2). In the roots, the 1/S value was 0.067, and a strong dominance of *Ilyonectria macrodidyma* (OTU 30) was found (Pi = 0.259), followed by *Penicillium* sp. (OTU

40, Pi = 0.111) and *Diaporthe columnaris*, *Plectosphaerella cucumerina*, and *Fusarium oxysporum* (OTUs 23, 27, 33, and 34; Pi = 0.074 each). All these dominant species were exclusively found in roots (Table 2). Conversely, *Alternaria* sp. (OTU 1), which was dominant in all the other tissues, was not found in roots (Table 2). In addition, *Epicoccum* sp. (OTU 4) was the most dominant in leaves and dominated in stems and cones, too, whereas it was absent in roots and scarcely present in male flowers. Among the other dominant species, *B. cinerea* (OTU 39) was found in cones only.

Considering the different localities, the highest S value was found in Piediluco, followed by Città di Castello and Belfiore; these localities also yielded the highest numbers of isolates (Table 3). Regarding the functional diversity, according to the FungalTraits database, most of the OTUs (36 out of 51, i.e., about 71%) are reported as plant pathogens, whereas the remaining 29% are saprotrophic of various substrates (Table S1). Also, most of the OTUs (29 out of 51) have endophytic interaction capability. In FungalTraits, the dominant genera are all reported as plant pathogens except *Penicillium*, but their endophytic capacity is also mentioned.

Table 3. Diversity of fungal strains in *H. lupulus* at different collection sites.

OTU No.	Taxon	Sampling Sites									
		P	**V**	**C**	**B**	**N**	**P**	**Bo**	**O**	**Ps**	**Pr**
1	*Alternaria* sp.	6	7	17	15	7	10	12		4	4
2	*Alternaria* sp.	1									
3	*Alternaria* sp.	1									
4	*Epicoccum* sp.	9	3	13	4	3	8				
5	*Alternaria infectoria*		1	2	1		1				
6	*Stemphylium vesicarium*	2					2				
7	*Bipolaris sorokiniana*			2				1			
8	*Periconia macrospinosa*				2						
9	*Pithomyces chartarum*				1						
10	*Neosetophoma italica*				1						
11	*Parastagonospora nodorum*						1				
12	*Periconia byssoides*									1	
13	*Pyrenophora tritici-repentis*			1							
14	*Neodidymelliopsis cannabis*					1					
39	*Botrytis cinerea*		2			5				1	
15	*Aureobasidium pullulans*	1			2	2	5				
16	*Cladosporium* sp.	4	2	7		2	4		1	3	1
17	*Mycosphaerella* sp.				1						
18	*Diplodia sapinea*								1		
19	*Diaporthe novem*	2	4		1	2	1				
20	*Diaporthe oncostoma*	3		2							
21	*Diaporthe* sp.		1				2			1	
22	*Diaporthe foeniculina*	1		2							
23	*Diaporthe columnaris*			1			1				
24	*Cytospora* sp.						1				
25	*Colletotrichum coccodes*			1	1					1	
26	*Colletotrichum gloeosporioides*			1						1	
27	*Plectosphaerella cucumerina*						2				
28	*Colletotrichum karsti*								1		
29	*Colletotrichum acutatum*						1				
30	*Ilyonectria macrodidyma*			4			3				
31	*Fusarium* sp.			1			2		2		
32	*Fusarium sambucinum*			3						2	
33	*Fusarium oxysporum*		1	2							
34	*Fusarium oxysporum*			1	1						
35	*Fusarium verticillioides*				1						
36	*Nemania serpens*	1									
37	*Truncatella angustata*			1							
38	*Nigrospora sphaerica*							1			
40	*Penicillium* sp.						3				
41	*Penicillium* sp.						1				
42	*Aspergillus* sp.					1					
43	*Talaromyces wortmannii*						1				
44	*Hyphodermella rosae*						1				
45	*Rhizoctonia solani*				1						

Table 3. *Cont.*

OTU No.	Taxon	Sampling Sites									
		P	**V**	**C**	**B**	**N**	**P**	**Bo**	**O**	**Ps**	**Pr**
46	*Itersonilia perplexans*				1						
47	*Vishniacozyma heimaeyensis*		1								
48	*Cryptococcus* sp.		1								
49	*Mucor fragilis*				1						
50	*Paraphoma* sp.						1				
51	*Sporormiella intermedia*			1							
	Total no. isolates	31	23	62	34	23	51	14	5	14	5
	Species richness	11	10	18	15	8	20	3	4	8	2

P = Ponte S. Giovanni; V = Valfabbrica; C = Città di Castello; B = Belfiore; N = Nera River; Pi = Piediluco lake; Bo = Bolsena Lake; O = Orvieto; Ps = Penna S. Giovanni; Pr = Potenza River.

3.3. Interaction Tests

To investigate endophytic fungi as potential biocontrol agents, we assessed their bioactivity against phytopathogenic fungi using two distinct methods. First, we employed a dual-culture technique, followed by evaluating the impact of non-volatile metabolites produced by the endophytes on the mycelial growth of the pathogens through an agar diffusion method.

Seven endophytic strains, namely E150 (*Epicoccum* sp., OTU 4), E157 (*Aureobasidium pullulans*, OTU 15), E545 (*Plectosphaerella cucumerina*, OTU 27), E149 (*Stemphylium vesicarium*, OTU 6), E343 (*Periconia byssoides*, OTU12), E536 (*Talaromyces wortmannii*, OTU43), and E425 (*Nigrospora sphaerica*, OTU38), were tested against the four pathogenic fungi (*Alternaria* sp., *Fusarium oxysporum*, *Botrytis cinerea*, and *Sclerotinia sclerotiorum*). The endophytic strains were selected among species not widely known as plant pathogens and as belonging to different phylogenetic groups. In particular, we focused on fungi belonging to the most represented classes (Dothideomycetes, Sordariomycetes, and Eurotiomycetes; see Table 2). Also, we selected both strains occurring in all the different plant tissues and strains with a different tissue specificity. For example, some strains (OTU4 and OTU15) are essentially ubiquitous, whereas others (OTU27, 12, 43, and 38) are tissue-specific.

Results of the interactions between endophytic and pathogen mycelia using both methodologies are shown in Table 4. All the endophytic strains showed antagonistic effects towards at least one pathogen. In the dual cultures, the inhibition appeared as a non-reciprocal contact between the endophyte and the pathogen, with the pathogen mycelium curling and growing slower than the control along the interaction line with the endophyte (Figure 2a–d). In the absence of inhibition, both fungi grew into each other without any visible signs of interaction (Figure 2e,f). Concerning the agar diffusion method, the percentages of inhibition are reported in Table 4. The highest percentage of inhibition was shown by *P. cucumerina* against *Alternaria* sp. (Figure 3a,b). An inhibition signal was evidenced also in the dual culture of these mycelia.

Table 4. In vitro antagonism of seven endophytic strains against four pathogenic fungi using dual-culture and crude extracts assays.

Isolate Name	**PATHOGENIC FUNGUS**			
	E140_OTU1_*Alternaria* sp.	E529_OTU33_*F. oxysporum*	AR593_*B. cinerea*	AR397s2_*S. sclerotiorum*
ENDOPHYTIC FUNGUS				
E150_OTU4_*Epicoccum* sp.	−/15.3	−/0	+/0	+/0
E157_OTU15_*Aureobasidium pullulans*	−/0	−/0	+/27.3	−/12.5
E545_OTU27_*Plectosphaerella cucumerina*	+/40.9	−/0	−/0	−/0
E149_OTU6_*Stemphylium vesicarium*	+/7.5	−/12.0	−/0	−/10.9
E343_OTU12_*Periconia byssoides*	+/0	−/6.5	−/0	+/8.97
E536_ OTU43_*Talaromyces wortmannii*	−/0	+/5.6	−/0	−/8.97
E425_OTU38_*Nigrospora sphaerica*	+/15.6	−/3.2	−/0	+/14.5

Results of the dual cultures are reported as "+" (endophytic fungus inhibition) or "−" (no inhibition), followed by percentages of pathogen growth inhibition in the crude extract assays.

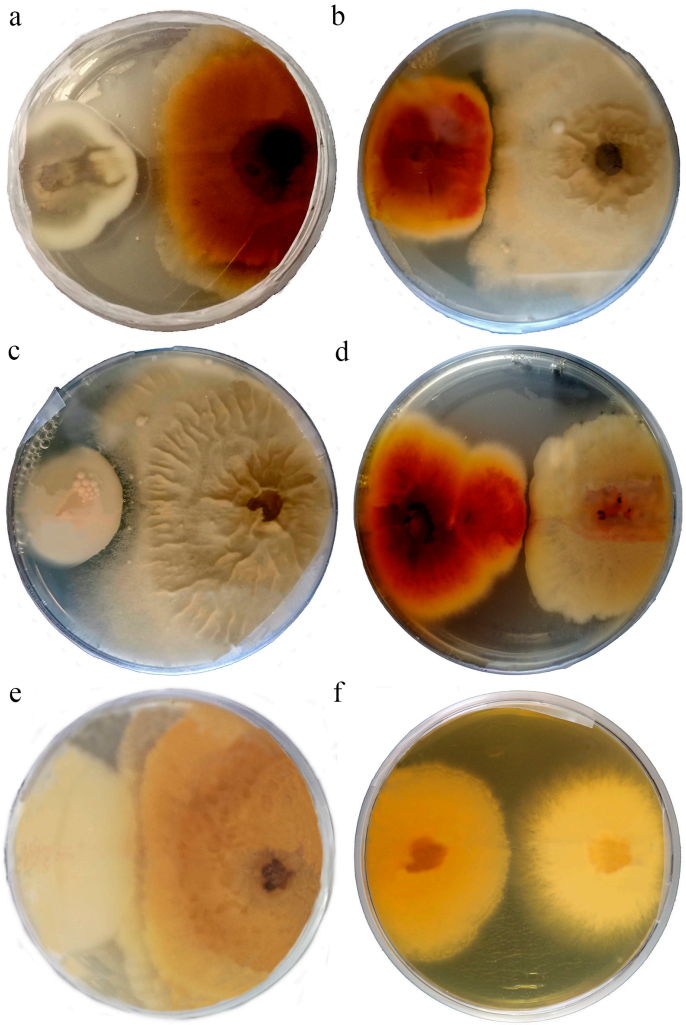

Figure 2. Dual-culture interaction tests between selected endophytic strains (left side of the plates) and pathogenic fungi (right side of the plates). (**a**) *Periconia byssoides* (E343) vs. *S. sclerotiorum* (AR397s2); (**b**) *Epicoccum* sp. (E150) vs. *B. cinerea* (AR593); (**c**) *A. pullulans* (E157) vs. *B. cinerea* (AR593); (**d**) *Epicoccum* sp. (E150) vs. *S. sclerotiorum* (AR397s2); (**e**) *A. pullulans* (E157) vs. *S. sclerotiorum* (AR397s2); (**f**) *Epicoccum* sp. (E150) vs. *F. oxysporum* (E529)**.**

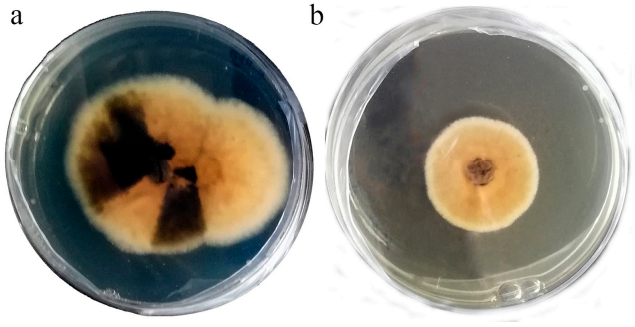

Figure 3. Agar diffusion method interaction test of the pathogenic strain *Alternaria* sp. (E140) growing in media added (**a**) or not (**b**) with culture broth of the endophyte E545.

4. Discussion

Wild relatives of crop plants are highly valued and exploited, compared to their domesticated counterparts, for genes that confer increased resistance to biotic and abiotic stresses. Wild plants host a wide range of microorganisms, including some beneficial

species that are absent or under-represented in the domesticated crops [28] and that provide such special traits for adaptation and resistance to stressing environmental conditions [29]. In fact, it has been largely reported that endophytes isolated from underutilized crops or their wild relatives exhibit higher diversity and richness than those found in related cultivars [30]. Furthermore, when introduced as bioinoculants, these endophytes aid cultivars in overcoming adverse conditions [31]. In this study, we exploited Italian wild hop accessions previously characterized in a population genetics study [32] to isolate and characterize their fungal endophytic communities living in the different plant tissues (roots, stems, leaves, and flowers). The fungal diversity was compared across ten wild productive sites in Central Italy. A biocontrol potential activity against common phytopathogenic fungi was unveiled for some of the isolated fungi. An ex situ conserved collection of endophytic fungal strains was established for further bioactivity tests.

4.1. Diversity of Hop Endophytic Fungi

The ITS region employed here as a barcode for the identification of endophytic fungi does not exhibit uniform variability across all fungal groups. Insufficient variability in the ITS region can pose challenges for species-level identification, particularly within certain species-rich genera of Ascomycota, such as *Alternaria*, *Aspergillus*, *Cladosporium*, *Penicillium*, and *Fusarium* ([33] and references therein). This is the reason why, in this work, species names were assigned to 37 out of the 51 OTUs, whereas 14 OTUs were identified at the genus level only. The large majority of fungal strains identified among the 51 OTUs belong to Ascomycota (88%), with a predominance of the Dothideomycetes and Sordariomycetes classes, whereas Basidiomycota and Mucoromycota represented the remaining 9.8 and 2%, respectively. The prevalence of Ascomycota among fungi colonizing both aboveground and belowground plant tissues is widely documented in different species [34], including other species of the Cannabaceae family [35]. Consistent with this, Ascomycota appear to possess superior adaptations compared to Basidiomycota and other phyla for colonizing internal plant tissues [36]. Interestingly, in the present work, Basidiomycota were absent in hop flowers and stems. Additionally, *Alternaria* sp. emerged as the dominant taxon in all tissues except for the roots, where it was completely absent. This fungus is among the most widespread plant endophytic species [37] and was previously reported as a dominant endophyte in different plant species [38,39]. *Alternaria alternata* is known to produce for the host plant the growth regulator indole-acetic acid [39], a key molecule for important physiological processes such as cell division or cell elongation, tissue differentiation, phototrophic or geotropic responses, and all subsequent effects on plant growth and development [40]. *Epicoccum* sp. dominated in leaves slightly more than *Alternaria* sp. *Epicoccum* spp. are ubiquitous ascomycetes known to produce diverse classes of biologically active secondary metabolites holding cytotoxic, anticancer, antimicrobial, and anti-diabetic activities [41]. As evidenced by the FungalTraits characterization, *Epicoccum* strains may be plant pathogens, saprotrophs, or endophytes. Some endophytic species of *Epicoccum* have been demonstrated to have biological control activity against various plant pathogens [42,43]. Given that certain *Epicoccum* strains exhibit dual roles as both pathogens and biological control agents, understanding the pathogenic potential of the fungal strain is of paramount importance [44]. *Ilyonectria macrodydima* was the most dominant species in roots and was exclusively found in this tissue. This fungus is not commonly found at the endophytic status; rather, it can cause root rot in olive [45] and black-foot disease in grapevine [46], a serious disease in most wine- and grape-producing regions of the world. In line with our findings, *I. macrodydima* was previously identified as the most abundant fungus in roots, even in wild grapevines [47].

Interestingly, these and most of the other dominant species (*Diaporthe* spp., *P. cucumerina*, *F. oxysporum*, and *B. cinerea*) are well-known phytopathogenic fungi. Indeed, concerning the lifestyle and trophic modes of the identified fungal genera, we observed that, according to the FungalTraits database, most of the OTUs (71%) belong to plant pathogenic genera as for their primary lifestyle. This outcome is unsurprising, given that we isolated fungi from wild accessions, which, being surrounded by several plant species, are likely to be significantly exposed to a greater diversity of fungi compared to cultivated hops. We are currently engaged in a dedicated research project to test this hypothesis further by comparing fungal communities associated to cultivated and wild hops through metabarcoding approaches. The abundance of pathogenic fungal species could also mean that, although we isolated mycelia from healthy tissues and adopted the classical procedures for the isolation of plant endophytic fungi, these strains may not strictly be endophytic. As Schulz and Boyle [48] suggested, the endophytic condition should be viewed as a temporary status since plant–endophyte interaction may change over time depending on several factors. Endophytic fungi may in fact behave as latent pathogens that, because of physiological changes in the host, such as abiotic stress, growing stages, and interaction with other microorganisms [49], might switch from a symptomless, endophytic condition to a pathogenic stage. In addition, virulence genes can be activated or deactivated by mutations [50]. For these reasons, despite the recognized beneficial effects of many of the isolated species, the occurrence of such a large number of potentially pathogenic fungi is somewhat concerning since it may indicate the emergence of phytopathogens in wild hops in Italian and, more broadly, Mediterranean environmental conditions. This could potentially jeopardize ongoing and future hop cultivations in these regions.

We observed a tissue-specific pattern for some taxa. Dothideomycetes were exclusively found in the aerial parts of the plants, primarily represented by Pleosporales. Conversely, roots were predominantly colonized by Sordariomycetes, particularly taxa belonging to Hypocreales. Moreover, roots shared only three out of fifteen OTUs with other tissues, whereas the other tissues shared more OTUs among each other. Several species showed tissue-specificity: considering the most abundant species, notable examples include *B. cinerea* in the cones, *I. macrodidyma* in the roots, *Bipolaris sorokiniana* in the leaves, and *Cladosporium* sp. in both leaves and cones. Such a tissue-specific pattern has been observed across various plant species [15,51], likely influenced by specific ecological challenges, such as phytopathogens and other biotic/abiotic stresses, encountered by the different plant organs. We would like to point out that the main goal of this study was to establish a strain collection aimed at identifying potential biocontrol agents. Therefore, the distribution of the different taxa in hop plants presented here is preliminary. Currently, a study utilizing a metabarcoding approach is underway to comprehensively investigate the overall biodiversity of hop endophytic fungi, encompassing variations across different sites, seasons, and plant tissues.

4.2. Antifungal Activities of the Isolated Strains

A major goal outlined by the European Union (EU) is the conservation of biodiversity for the planet health. This is pursued through measures such as strengthening protected areas, restoring degraded ecosystems via the promotion of organic farming and reforestation, and ensuring sustainability in food production. Therefore, the directives of the European Green Deal aim to reduce the reliance on pesticides, antimicrobials, and fertilizers in agriculture to combat plant diseases. Instead, there is a prioritization of biological control methods over synthetic compounds. Hence, particular attention has been paid to the endophytic fungal diversity of crop plants for 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 [13]. In this study, a preliminary

screening of the biocontrol potential of the fungal strains isolated from hop was performed using four distinct phytopathogenic fungi as target species. *Alternaria* sp. and *F. oxysporum* were chosen, as they are common pathogens affecting various plant species, including the potential threat they pose to *H. lupulus*, as indicated by our findings. Additionally, *B. cinerea* and *S. sclerotiorum* were included in the tests owing to their widespread presence as plant pathogens known to impact *H. lupulus* as well, where they can induce grey mold disease in hop cones, especially in heavy rainfall seasons. This species is also known as a primary cause of post-harvest infections of numerous agricultural commodities. Consequently, the biocontrol of *B. cinerea* holds significance not only during cultivation but also in the stages of harvesting and distributing the final product. Notably, we utilized strains of these species previously isolated from diseased leaves of *Vitis vinifera*. The fungal strains tested against the pathogens were selected from different classes and orders and from different plant tissues. In most cases, there was consistency between the results obtained from the two different inhibition assays. They both either showed positive or negative reactions of the endophyte towards the pathogen (Table 4). However, it is worth noting that in 10 out of the 28 endophyte/pathogen combinations, the two tests yielded contrasting results. In some instances, inhibition was observed with the agar diffusion method but not with the dual-culture method. This suggests that under in vitro optimal growing conditions, reciprocal contact between the mycelia may not be sufficient to elicit an inhibition reaction. Instead, achieving inhibition may require a high concentration of bioactive molecules produced by the endophyte over an extended period (two weeks in our experiments), regardless of the presence of the pathogen. On the contrary, the detection of inhibition signals exclusively in co-culture conditions suggests that the presence of the pathogen stimulates the endophyte to synthesize bioactive compounds. The most susceptible pathogenic fungi, inhibited by nearly all the tested endophytes, were *S. sclerotiorum* and *Alternaria* sp. This evidence suggests the opportunity to individually test these endophytes and their consortia, both under greenhouse and field conditions, to evaluate their potential as biocontrol agents for plants challenged with *S. sclerotiorum* or *Alternaria* spp. Such experiments will enable the identification of suitable SynComs for the management of plant diseases. Among the tested interactions, the strongest inhibition signal was evidenced by the growth medium of *P. cucumerina* against *Alternaria* sp., with a 41% growth inhibition. The dual-culture method confirmed this result. *P. cucumerina* is widely known as a plant pathogen capable of causing sudden death and blight disease in a variety of crops [52] rather than as an endophyte with potential protective properties against other fungal pathogens. Interestingly, nematocidal [53] and antibacterial [54] properties have been reported for *P. cucumerina*. Thus, our evidence is quite unexpected and points to the possible new potential of this species in the biological control of plant diseases caused by pathogenic fungi. Additionally, *A. pullulans* showed another significant bioactive role against *B. cinerea* as evidenced by both methods, although *B. cinerea* was the least susceptible pathogen among those tested against our panel of endophytic fungi. *A. pullulans* is a ubiquitous saprophytic, yeast-like fungus with a high biotechnological potential. It has been reported as an antifungal agent against post-harvest pathogens of fruit and vegetables (e.g., *B. cinerea*) [55] and an effective biocontrol agent against airborne plant pathogens. All these attributes are of major relevance to the vitivinicultural sector [56] and could potentially be leveraged in the emerging hop sector as well. *Epicoccum* is among the dominant species in the hop plants analyzed, with the 40 strains isolated from different tissues belonging to a single OTU. One of these strains exhibited bioactivity against *B. cinerea* and *S. sclerotiorum* using the dual-culture method and against *Alternaria* sp. using the agar diffusion method. Previous studies demonstrated that certain endophytic species of *Epicoccum* possess biological control activity against various plant pathogens, including *B. cinerea*, *S. sclerotiorum*, and *Alternaria* sp. However, in

plant ecosystems, *Epicoccum* species can function as endophytes, saprophytes, or pathogens. Therefore, it is advisable to assess the pathogenic potential of an *Epicoccum* isolate before evaluating its effectiveness as a biological control agent against a specific pathogen in order to prevent disease development and minimize plant yield losses [43]. The other tested strains, including *S. vesicarium*, *P. byssoides*, *T. wortmannii*, and *N. sphaerica*, showed potential antagonisms against the tested pathogenic fungi. Although some antifungal activities were previously reported for these species [57–59], to the best of our knowledge, they were tested against different pathogenic fungi compared to those used in this study.

Overall, the preliminary results obtained in this work regarding the biological activities of the isolated fungi suggest that these strains could be novel resources of antifungal metabolites to be exploited in the sustainable management of crop diseases not only in the emerging hop sector in Italy but also in other agricultural sectors. Moving forward, our future endeavors will focus on exploring the biocontrol potential of additional fungal strains isolated in this study. Moreover, it will be interesting to disclose the molecular mechanisms and the specific metabolites involved in these biological activities. For example, this could involve whole-genome sequencing and transcriptome analyses of selected endophytic fungal species. As an example, these studies could be focused on Eurotiomycetes fungi, such as *Talaromyces* spp., which are well known to produce secondary metabolites with antibacterial activity [60,61] but scarcely known for their antifungal potential.

5. Conclusions

To our knowledge, this is the first comprehensive characterization of endophytic fungal communities of the different tissues of wild hop (*H. lupulus*) plants. Interaction tests allowed us to identify interesting strains with a biocontrol activity on phytopathogenic fungi. All the isolated strains were conserved ex situ for further bioactivity tests and production of metabolites of agronomical and biotechnological interest. This paper provides original data with a key relevance for the environmentally friendly management of plant diseases.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/d17020094/s1. Table S1: FungalTraits.

Author Contributions: Conceptualization, C.R., A.R. and B.B.; methodology, A.R.; formal analysis, A.R. and M.C.; investigation, C.R., A.R., B.B. and M.C.; writing—original draft preparation, C.R.; writing—review and editing, A.R and B.B. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: An ex situ conserved collection of endophytic fungal strains was established at CNR-IBBR-Perugia as part the BioMemory Project (https://biomemory.cnr.it/collections/CNR-IBBR-FABI). Sequence data were submitted to GenBank (Accession no. OQ257045 to OQ257306).

Conflicts of Interest: The authors declare no conflicts of interest.

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