

Article

Novel Endophytic *Trichoderma* spp. Isolated from Healthy *Coffea arabica* Roots are Capable of Controlling Coffee Tracheomycosis

Temesgen Belayneh Mulaw^{1,2}, Irina S. Druzhinina¹, Christian P. Kubicek¹ and Lea Atanasova^{1,*}

¹ Research Area Biotechnology and Microbiology, Institute of Chemical Engineering, Vienna University of Technology, Vienna 1060, Austria; E-Mails: tmulaw@umces.edu (T.B.M.); druzhini@mail.zserv.tuwien.ac.at (I.S.D.); ckubicek@mail.zserv.tuwien.ac.at (C.P.K.)

² Center for Environmental Science, Institute of Marine and Environmental Technology, University of Maryland, Baltimore, MD 21201, USA

* Author to whom correspondence should be addressed; E-Mail: lea.atanasova@tuwien.ac.at; Tel.: +43-1-588-0116-6571; Fax: +43-1-588-011-7299.

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Abstract: One of the biggest threats to coffee growers in East Africa are emerging vascular wilt diseases (tracheomycosis) caused by *Fusarium* spp. Many *Trichoderma* species are known to be natural antagonists of these pathogens and are widely used in biological control of fungal plant diseases. More recently, several *Trichoderma* spp., which exhibited high antifungal activity have been isolated as endophytes. Consequently, we have investigated the presence and the antagonistic activity of endophytic *Trichoderma* isolated from roots of healthy coffee plants (*Coffea arabica*) from the major coffee growing regions of Ethiopia. Our results showed that community of *Trichoderma* spp. in roots of *C. arabica* contains fungi from coffee rhizosphere, as well as putatively obligate endophytic fungi. The putatively “true” endophytic species, until now, isolated only from coffee plant ecosystems in Ethiopia and recently described as *T. flagellatum* and novel *T. sp.* C.P.K. 1812 were able to antagonize *Fusarium* spp., which cause coffee tracheomycosis. Moreover, we found that strains of these species are also highly antagonistic against other phytopathogenic fungi, such as *Alternaria alternata*, *Botryotinia fuckeliana* (anamorph: *Botrytis cinerea*), and *Sclerotinia sclerotiorum*.

Keywords: biocontrol; tracheomycosis; *Trichoderma flagellatum*; *Fusarium* sp.; coffee

1. Introduction

Endophytic fungi are defined as organisms colonizing healthy plant tissue without causing overt symptoms of apparent injury to the host [1]. They have been isolated from nearly all plants families growing in different climatic regions of the world [2]. Many commercially relevant arboreous plants, crops and officinal herbs support communities of endophytic fungi [3–5]. Although being biotrophic and consume plants nutrients, endophytic fungi may exhibit a beneficial role for plant health antagonizing pests via mycoparasitism, competition, and/or antibiosis. In addition they may directly stimulate plant growth and immune response inducing resistance to diseases [6–8].

Coffea arabica is considered a native plant in Ethiopia and coffee beans are the country's largest export commodity [9,10]. One of the biggest threats to coffee growers world-wide are emerging fungal wilt diseases, in particular tracheomycosis caused by *Gibberella xylarioides* (anamorph *Fusarium xylarioides*) [11]. In Ethiopia incidences of tracheomycosis are reported to concern 60% of total crops, and are accompanied by significant yield losses due to severe damage and ultimate death of millions of coffee bushes [12]. Integrated Disease Management programs would be of a great potential to combat *Fusarium* pests in Ethiopia.

The fungal genus *Trichoderma* (teleomorph *Hypocrea*, Ascomycota, Dikarya) contains some of the most potent biocontrol agents used today [13,14]. In addition, it has been shown that several of its taxa occur as endophytes particularly in the tropical arboreous plants, and that the strains often exhibit high antagonistic activity against pathogens of these plants [15–18]. However, the importance of *Trichoderma* or other endophytic fungi as a part of endogenous microbial diversity and their application in the biological control of tracheomycosis on the coffee plants has not been investigated yet.

The objective of this study was to determine the endophytic fungal community of healthy roots of *C. arabica* with particular emphasis on the presence of *Trichoderma* and its mycoparasitic potential against *Fusarium* causing tracheomycosis.

2. Methods

2.1. Root Samples and Isolation of Endophytic Fungi

Roots of *C. arabica* were collected from different sites of four major coffee growing agroecological systems of Ethiopia (for details see [19]) during the wet season (August 2006) and were kept in paper bags at +4 °C until the examination. Five samples were randomly selected from the mixed sample set and processed for the isolation of endophytic fungi according to the protocol of Mysore *et al.* [20]. Briefly, roots were cleaned under running tap water to remove debris and were then air-dried on a sterile filter paper. From each sample, 1 cm segments of root slices were surface-sterilized by immersing them in 95% ethanol (2 min), followed by sodium hypochlorite (4% available chlorine; 5 min), and were then washed three times with sterile water. Samples were then allowed to dry on a sterile filter paper for 10 min in a sterile laminar flow chamber. Segments were placed horizontally on potato dextrose agar (PDA) supplemented with streptomycin sulphate (0.4 mg/mL). In a course of 15 days incubation at 25 °C, individual hyphal tips of the developing colonies were re-plated onto PDA plates and were further incubated for 8–10 days. Eventually, single spore cultures for each strain were prepared to assure sample

purity. Isolates are stored in 50% glycerol at -80°C in the Collection of Industrial Microorganisms of Vienna University of Technology (TUCIM, Vienna, Austria).

2.2. DNA Extraction and PCR Amplifications

For DNA extraction mycelium was cultivated three days on 3% MEA (malt extract agar). Genomic DNA was extracted using Qiagen DNeasy Plant Minikit (Hilden, Germany) according to the manufacturer's instructions. Internal transcribed spacer region (ITS1 and 2) of the rRNA gene cluster was amplified using the primers SR6R and LR1 [21] as described by Kullnig-Gradinger *et al.* [22]. Additionally, the amplification of approximately 1.4 kb fragment of translation elongation factor 1- α (*tef1*) using primers EF1728F [23] and TEF1LLerev (5'-AAC TTG CAG GCA ATG TGG-3') was performed as described previously (see e.g., [24]). Template DNA was purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and was subjected to automatic sequencing at Eurofins MWG Operon (Ebersberg, Germany).

2.3. DNA Barcoding

Analysis of ITS1 and 2 sequences was performed using sequence similarity search tool (blastn) against the NCBI GenBank database [25]. *Trichoderma* ITS1 and 2 sequences were subjected to the oligonucleotide barcode program *TrichOKey* [26]. Still, ambiguous cases were verified by the analysis of the 4th intron of the translation elongation factor 1- α -encoding gene (*tef1*) using sequence similarity search against NCBI GeneBank and *TrichoBLAST* [27] databases. Identification of *Fusarium* strains was done based on the analysis of 690 bp fragment of translation elongation factor 1- α (*EF-1a*) gene amplified using EF1 and EF2 primer pairs as described by O'Donnell *et al.* [28]. Sequences were submitted to similarity search tools against FUSARIUM-ID [29] and NCBI GenBank databases. The NCBI accession numbers for sequences obtained in this study are listed in Tables 1 and 2.

Table 1. List of endophytic fungi (without *Trichoderma* spp.) isolated from the roots of *C. arabica* and their identification based on *tef1* barcode.

TUCIM No. (C.P.K)	GenBank Acc. No	Identification				
		% similarity/% query coverage	Phylum	Putative identification	The most similar NCBI GenBank entry	
					taxon	Acc. No
3484	FJ827622	100/100	Ascomyc.	<i>Aspergillus</i>	<i>Aspergillus flavus</i>	AM745114
3485	FJ827626	100/94		<i>Cladosporium</i>	<i>Cladosporium cladosporioides</i>	EF577236
3478	FJ827623	100/98		<i>Rhizopycins</i>	<i>Rhizopycnis</i> sp. IBL 03177	DQ682600
3078	FJ827619	99/94		<i>Penicillium</i>	<i>Penicillium</i> sp. RCEF3398	EF570358
3084	FJ827620	99/95				
3468	FJ827620	99/92		<i>Phomopsis</i>	<i>Phomopsis columnaris</i>	AF439625
3471	FJ827629	99/92				
3473	FJ827628	99/93				
3512	FJ827630	94/98		Dipodascaceae	<i>Dipodascus australiensis</i>	AF157596
3474	FJ827625	99/98		<i>Macrophomina</i>	<i>Macrophomina phaseolina</i>	EU250575

Table 1. Cont.

TUCIM No. (C.P.K)	GenBank Acc. No.	Identification			
		% similarity/% query coverage	Phylum	Putative identification	The most similar NCBI GenBank entry
					taxon Acc. No.
3486	FJ827624	100/97		Pleosporales	Pleosporales sp. IBL 03175 DQ682598
3337	FJ827616	99/99		<i>Fusarium equiseti</i>	<i>F. equiseti</i> isolate 45/1.2.1 DQ854855
3469	FJ827615	99/98			
3514	FJ840530	99/98			
3466	FJ827613	99/98			
3472	FJ827614	99/98			
3513	n/a	99/98			
3465	FJ840527	99/98			
3480	FJ827612	93/99			
3470	FJ840525	93/98			<i>F. equiseti</i> isolate SAT73 DQ465946
3479	FJ827610	93/99			<i>F. equiseti</i> isolate 21/1.2 DQ854851
3332	FJ840526	99/98			
3509	FJ827611	99/98		<i>F. solani</i>	<i>Fusarium</i> sp. NRRL 43704 FSSC EF453029
3510	FJ840531	99/98			
3511	FJ840532	99/98			
3330	FJ840533	99/98			
3515	FJ840529	99/99		<i>F. oxysporum</i>	<i>F. oxysporum</i> isolate SAT77 DQ465933
3476	FJ827617	99/99			
3475	FJ827618	98/100	Basidiomyc.	<i>Trametes</i>	<i>Trametes versicolor</i> FJ608587

Table 2. Endophytic *Trichoderma* strains isolated from roots of *C. arabica*.

TUCIM No. (C.P.K)	NCBI GenBank		TUCIM No. (C.P.K)	NCBI GenBank		TUCIM No. (C.P.K)	NCBI GenBank	
	ITS1 and 2	<i>tef1</i>		ITS1 and 2	<i>tef1</i>		ITS1 and 2	<i>tef1</i>
Section <i>Trichoderma</i>			<i>Harzianum</i> - <i>Catoptron</i> Clade			Section <i>Longibrachiatum</i>		
<i>Trichoderma hamatum</i>			<i>Hypocrea</i> ‘ <i>pseudoharzianum</i> ’ nom. prov.			<i>T. flagellatum</i>		
3331	FJ461539	n/a	3440	FJ461557	FJ763164	3334	FJ461542	FJ763149
3333	FJ461540	FJ763148	3441	FJ461558	FJ763165	3345	FJ461551	FJ763158
3335	FJ461541	n/a	3442	FJ461559	FJ763166	3350	FJ461556	FJ763163
3336	FJ461543	FJ763150	3443	FJ461560	FJ763167	3496	FJ461568	FJ763174
3338	FJ461544	FJ763151	3501	FJ461573	FJ763177	3503	FJ461575	FJ763179
3342	FJ461548	FJ763155	3502	FJ461574	FJ763178	3504	FJ461576	FJ763180
3343	FJ461549	FJ763156	3506	FJ461578	FJ763181	3517	FJ461580	FJ763182
3346	FJ461552	FJ763159	<i>T. sp.</i> C.P.K. 1812			3522	FJ461585	n/a
3347	FJ461553	FJ763160	3328	FJ461537	FJ763146	3523	FJ461586	n/a
3348	FJ461554	FJ763161	3329	FJ461538	FJ763147	3524	FJ461587	FJ763183

Table 2. Cont.

TUCIM No. (C.P.K)	NCBI GenBank		TUCIM No. (C.P.K)	NCBI GenBank	
	ITS1 and 2	<i>tef1</i>		ITS1 and 2	<i>tef1</i>
Section <i>Trichoderma</i>			<i>Harzianum-Catoptron</i> Clade		
<i>Trichoderma hamatum</i>			<i>Hypocrea</i> ‘ <i>pseudoharzianum</i> ’ nom. prov.		
3491	FJ461563	n/a	3444	FJ461561	FJ763168
3492	FJ461564	FJ763170	3490	FJ461562	FJ763169
3493	FJ461565	FJ763171	3494	FJ461566	FJ763172
3497	FJ461569	n/a	3495	FJ461567	FJ763173
3505	FJ461577	n/a	3498	FJ461570	n/a
3507	FJ461579	n/a	3499	FJ461571	FJ763175
3518	FJ461581	n/a	3500	FJ461572	FJ763176
3519	FJ461582	n/a	<i>T. sp. C.P.K. 1833</i>		
3520	FJ461583	n/a	3339	FJ461545	FJ763152
3521	FJ461584	n/a	3341	FJ461547	FJ763154

2.4. Phylogenetic Analyses

For phylogenetic analysis DNA sequences were aligned with the CLUSTAL X program (version 1.81) [30] and visually verified with GeneDoc software (version 2.6) [31]. Phylogenetic analysis was performed using the maximum-parsimony method (observed p-distance, no evolutionary modeling required) implemented in PAUP*4.0b10 applying a heuristic search ($n = 1000$) with the random addition of sequences and the TBR tree-swapping algorithm. The reliability of the obtained clades was tested by 500 bootstrap replications. Bootstrap values of >74% were considered significant. The phylogenetic position of the isolates attributed to the *Harzianum-Catoptron* Clade was inferred using the Bayesian phylogenetic method implemented in MrBayes v.3 [32] with 3,000,000 mcmc generations and a general time-reversible model for nucleotide substitutions [33], as described previously [34]. Posterior probability above 0.94 was considered significant [35].

2.5. Screening for Antifungal Activity

Antifungal activity of endophytic *Trichoderma* isolates was tested against the causative agents of coffee tracheomycosis, *G. xylarioides*, and *G. jujikuroi* (anamorph *Fusarium oxysporum* or FOX later on in the ms), and also against three other plant pathogenic fungi, *Alternaria alternata* (TUCIM 199), *Botryotinia fuckeliana* (anamorph: *Botrytis cinerea*, TUCIM 200) and *Sclerotinia sclerotiorum* (TUCIM 201). The corresponding isolates of *Fusarium* spp. were obtained from infected coffee roots collected in the same region. Antagonistic ability of *Trichoderma* strains against *G. xylarioides* and FOX was tested, as described by Ortiz and Orduz [36]: a 5 mm diameter agar plug was removed from a freshly prepared PDA plate and replaced by an equally sized plug from a seven days old culture of *G. xylarioides*, or FOX pre-grown on PDA. After incubation for a further four days an agar plug containing mycelium of the respective *Trichoderma* isolate (5 mm diameter) was placed on the plate, 3 cm distant from the *Fusarium* colony. The plates were then further incubated for five days.

Six replicates were set for each confrontation and plates containing two plugs of *Fusarium* culture were used as a control. Center-wise growth of *Trichoderma* and *Fusarium*, indicative by an internal mycelial growth radius (internal halo) and edge-wise growth indicated by a free mycelial growth radius (external halo), was also measured.

Dual confrontation assays with *A. alternata*, *B. cinerea* and *S. sclerotiorum* were made on PDA plates incubated for 10 days in a 12 h illumination cycle at 25 °C. Fresh culture agar plugs of prey and predator fungi were placed on a PDA plate located on opposite poles, each, 1 cm from the edge of the plate. Antagonistic potential was estimated as a reduction of the prey growth rate corrected for the growth rate of a prey when confronted with itself. To reveal the differences in mycoparasitic potential between endophytic and saprophytic *Trichoderma* sp. the results of endophytic strains isolated in this study were compared to the dual confrontation tests of saprophytic *Trichoderma* strains from TUCIM database (TU Collection of Industrial Microorganisms, TU Vienna). Antagonistic potential was presented as averaged growth inhibition or overgrowth of each plant pathogen (prey) by *Trichoderma* strains expressed in percentage. In both cases 100% represented unrestricted growth of the prey corrected for the confrontation with itself. Data were analyzed using Bartlett's test to verify the homogeneity of variances, and then subjected to one-way ANOVA using SPSS 17.0 software. Least significant difference (LSD) was used to compare treatment means.

3. Results

3.1. The Endophytic Fungal Community in Roots of *C. arabica*

A total of 300 segments from five healthy *C. arabica* roots were used for this study, of which 80 fungal isolates were recovered and tentatively identified at the generic or higher taxonomic level (Table 1) with aid of ITS1 and 2 rRNA barcodes. Eight genera and two families were assigned to the phylum Ascomycota, while a single Basidiomycota strain was identified as *Trametes* spp. Members of the genera *Trichoderma* (n = 50) and *Fusarium* (n = 18) were most frequently isolated.

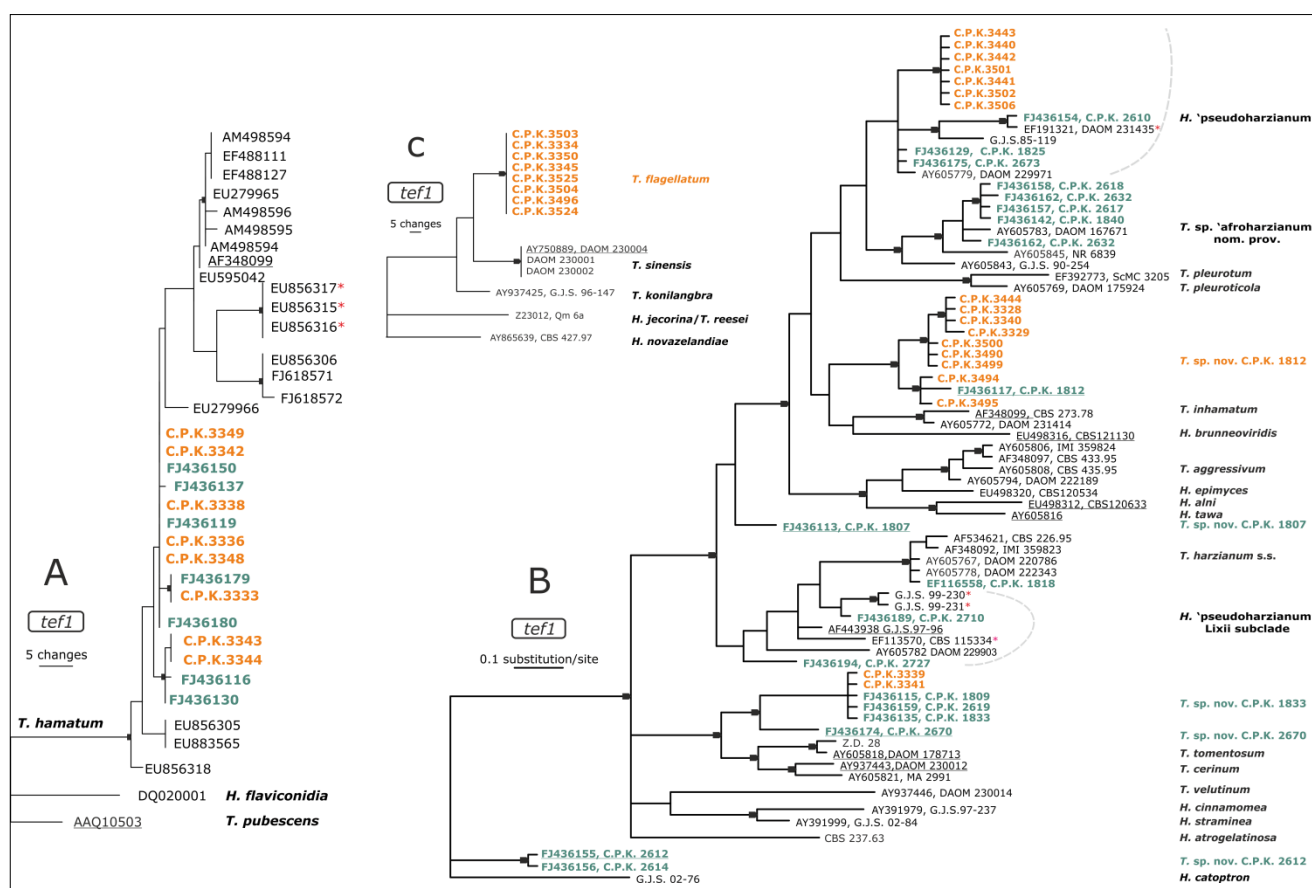
3.2. Diversity of Endophytic *Trichoderma*

ITS1 and 2 barcoding identified 21 strains as *T. hamatum*. In order to test whether they are identical to the *T. hamatum* populations which occur in coffee rhizosphere [19], we also sequenced a fragment of the elongation factor 1-alpha gene (*tef1*) containing the long (4th) intron. Phylogenetic analyses showed that *T. hamatum* isolates exhibited the same *tef1* allele as the strains previously found in rhizosphere of *C. arabica* [19] (Figure 1A). Genetic distances of Ethiopian *T. hamatum* strains to the type strain and other African isolates (from Cameroon, indicated by * on Figure 1A) suggest biogeographical isolation of the Ethiopian population associated with *C. arabica*.

The next majority of strains (18) were identified as members of the *Harzianum-Catoptron* Clade [37]. The phylogenetic analysis of *tef1* locus revealed that seven strains formed a separated clade inside of “*T. harzianum* species complex” (*H. ‘pseudoharzianum’* sensu Druzhinina *et al.* [36]), possessing a unique *tef1* allele (Figure 1B), that was not detected in rhizosphere of *C. arabica* [19]. Nine other isolates were identified as further members of a putative new species *T. sp.* C.P.K. 1812 (Figure 1B), which was so far only known from a single isolate from coffee rhizosphere [19]. The remaining two

isolates C.P.K. 3339 and 3341 belonged to the putative new phylogenetic species *T. sp. C.P.K. 1833* (Figure 1B), which also has been abundantly found in the rhizosphere of *C. arabica* in Ethiopia [19]; its formal taxonomic description based on multiloci phylogeny will be published elsewhere.

Figure 1. Phylogeny of endophytic *Trichoderma* strains isolated from coffee (*Coffea arabica*) roots inferred by maximum parsimony (MP) (A,C) and Bayesian (MB) analyses (B). Nodes supported by bootstrap values for MP of >74% or posterior probabilities for MB>0.94 respectively are indicated by black arrows. The color code indicates isolates associated with *C. arabica* as green for strains that were isolated from the coffee rhizosphere [19] and orange for endophytic strains isolated in this study. Sequences of established type cultures are underlined; asterisks mark strains of African origin. (A) *T. hamatum*, (B) *Harzianum-Catoptron* Clade, and (C) section *Longibrachiatum*.



By the means of ITS1 and 2 barcode, 11 strains could only be identified to the section level, and were attributed to the section *Longibrachiatum*. Furthermore, no polymorphism was found among their ITS1 and 2 and *tef1* sequences. A phylogenetic analysis of the *tef1* locus grouped them in a sister clade next to *T. sinensis* (Figure 1C), which was described based on three isolates from Taiwan [38]. Based on our isolates Samuels *et al.* [39] recently described this species as *T. flagellatum* Mulaw, Kubicek et Samuels as a common endophyte in roots of coffee in Ethiopia. It forms a clade with *T. sinense*, *T. konilangbra*, and *T. gillesii* [39,40].

3.3. Diversity of Endophytic *Fusarium* Isolates

We assessed the diversity and species composition of endophytic *Fusarium* spp. from *C. arabica*. To reveal the endophytic community of *Fusarium* isolates we sequenced and analysed the gene encoding for elongation factor 1- α (abbreviated as EF-1 α in *Fusarium*-related publications). The sequences were subjected to the NCBI GeneBank similarity search (blastn; [25]), and the 20 best hits were retrieved for the evaluation. By these means, the 18 *Fusarium* isolates were identified as four species with identical EF-1 α alleles. One of them appeared to be a member of the *Haematonectria haematococca* (anamorph *F. solani*) species complex, yet differing by one SNP at a position not found in any other query sequence NCBI GenBank for June 2011. The most similar hits were *G. jujikuroi* and *F. falciforme* strains from soil in South Africa, Tanzania, Mexico, and the Philippines (Table 1). Two other groups were identified as members of the *Gibberella intricans* (anamorph *F. equiseti*) species complex by means of 99 and 93% similarity (Table 1), whereas the fourth group with two isolates belonged to FOX species complex (Table 1). Based on the result of Bayesian phylogenetic analysis (data not shown) their sequences were cospecific with the most abundant EF-1 α phylotype also found in the rhizosphere of *C. arabica* in Ethiopia (T.B. Mulaw, I.S. Druzhinina, ms in preparation).

3.4. Antifungal Activity of *Trichoderma* Species

The antifungal activities of selected endophytic *Trichoderma* strains were first tested against the two agents of coffee tracheomycosis, FOX, and *G. xylarioides* respectively (Figures 2 and 3). All of the isolates strongly inhibited growth of both *Fusarium* spp., yet the antagonistic activity was strain specific. The inhibition of *G. xylarioides* growth by endophytic *Trichoderma* isolates appeared to vary as antibiosis (Figure 2) and coiling were not observed for all strains. In contrast, inhibition of FOX growth was consistent for all tested *Trichoderma* strains, being considerably weaker, mostly moderate, if compared to the inhibition of *G. xylarioides*. Strain C.P.K. 3340, belonging to the putative new species *T. sp.* C.P.K. 1812, exhibited the strongest antagonistic potential against both *Fusarium* species tested (Figures 2 and 3).

In order to test whether the mycoparasitic activity of endophytic *Trichoderma* strains is prey-specific or the mycoparasitism is generally increased for these strains, we made the dual confrontation assays with the three model plant pathogenic fungi *A. alternata*, *B. cinerea*, and *S. sclerotiorum* (Table 3). The data were evaluated in respect to 53 and 250 saprotrophic strains of *T. hamatum* and *H. 'pseudoharzianum'* ($\equiv$ former *T. harzianum* sensu lato; see Druzhinina *et al.* [37]), respectively, stored in TUCIM collection. Our data showed that the mycoparasitic potential of the endophytic strains is either equal or higher to the potential of saprotrophic *T. hamatum* and *H. 'pseudoharzianum'*. Interestingly, the mode of antagonistic action in novel putative species seems to be species specific; *T. sp.* C.P.K. 1812 showed superior ability to overgrow *B. cinerea* and *S. sclerotiorum*, while more than 50% of *T. flagellatum* strains failed to overgrow any of the three plant pathogens (Table 3). The endophytic strains of *T. hamatum* are essentially more active against *B. cinerea* than saprotrophic strains used in this study. Furthermore, all *T. hamatum* strains exhibited significantly higher potential to overgrow *S. sclerotiorum* compared to other species. However, all endophytic *Trichoderma* species found in this survey revealed high antagonistic potential and can well inhibit prey's growth.

Figure 2. Antagonistic potential of selected *Trichoderma* strains against *G. xylarioides* and *G. jujikuroi*. The shadowing indicates the two isolates capable of producing a diffusible inhibitor against *Fusarium* (antibiosis). Black and white bars indicate the inhibition of radial growth for *G. xylarioides* and *G. jujikuroi* respectively.

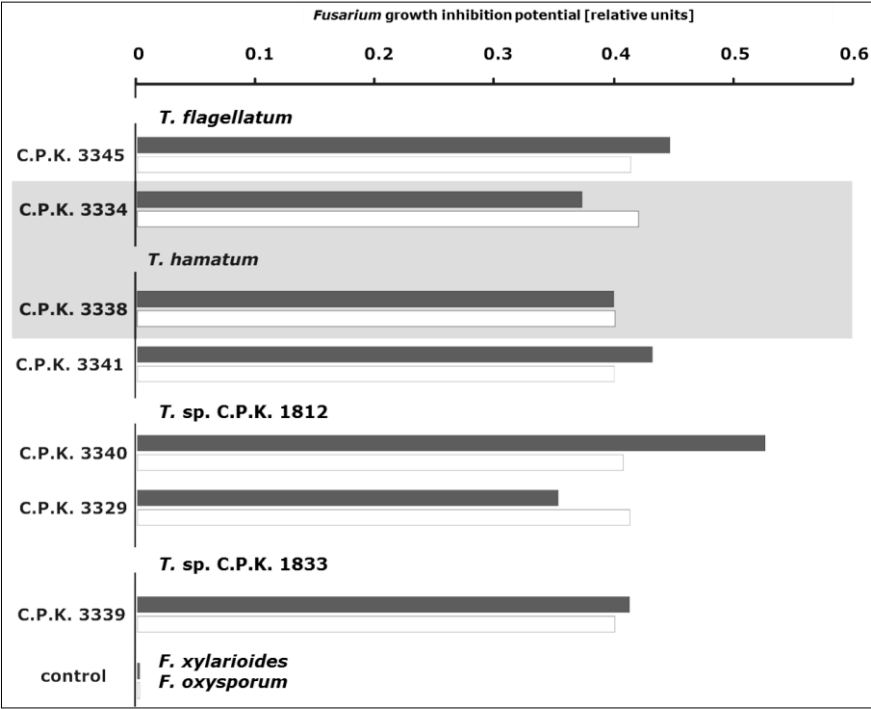


Figure 3. Dual confrontation plates with *Trichoderma* strains (always above) against four plant pathogens (always below). White lines show the extension of FOX colony.

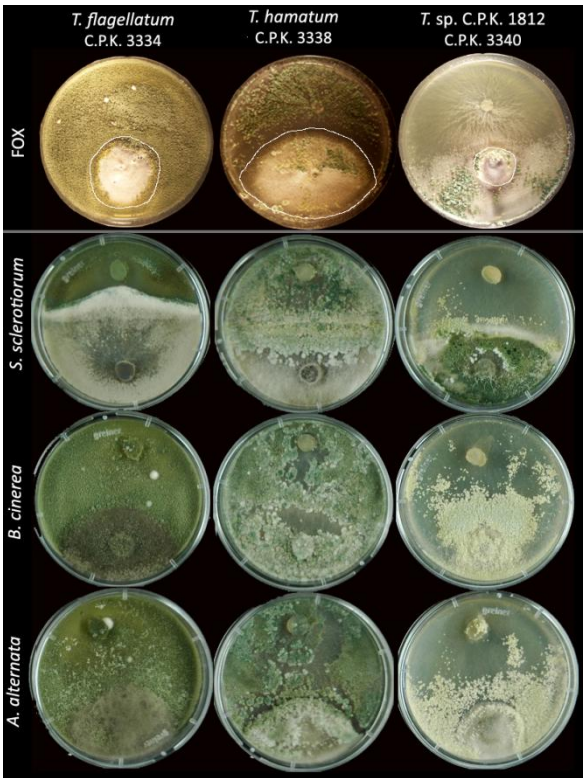
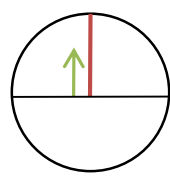


Table 3. Antagonistic potential (plant pathogen's growth inhibition and overgrowth) of endophytic *Trichoderma* strains isolated from *C. arabica* roots and their comparison to saprotrophic *T. hamatum* and *H. 'pseudoharzianum'* nom. prov.

	Inhibition of preys (% *)									% of strains capable to overgrow the prey						
	Per prey species							For 3 preys			Per prey species				For 3 preys	
	N	Aa		Bc		Ss		N	AV	SD	N (=100%)	Aa	Bc	Ss	N (=100%)	
		AV	SD	AV	SD	AV	SD									
Endophytic strains from roots of <i>C. arabica</i>																
<i>T. hamatum</i>	19	33.0	1.19	43.37	2.67	10.53	0.65	57	36.28	17.66	19	63.2	89.5	89.5	57	84.2
<i>H. ‘pseudoharzianum’</i> nom. prov.	6	38.3	8.67	35	6.18	13.17	2.32	18	35.5	16.74	6	16.7	66.7	66.7	18	62.5
<i>T. sp. C.P.K. 1812</i>	10	31.5	1.37	38.3	6.81	12.9	1.01	30	34.65	16.28	10	20.0	100.0	100.0	30	80.0
<i>T. flagellatum</i>	11	39.9	2.2	38.55	9.56	10.67	2.78	33	38.29	19.49	11	18.2	27.3	45.5	33	45.2
<i>T. sp. C.P.K. 1833</i>	18	36.2	1.42	40.06	7.73	11.39	1.03	54	35.17	16.05	18	27.8	61.1	88.9	54	59.7
Control (saprotrophic strains)																
<i>T. hamatum</i>	53	36.19	7.17	31.25	8.47	8.45	0.95	159	31.75	17.12	53	69.8	88.7	75.5	159	82.3
<i>H. ‘pseudoharzianum’</i> nom. prov.	250	35.76	5.56	30.38	6.26	9.11	1.21	750	30.61	16.22	250	45.2	87.6	83.6	750	78.1

* 100% is unrestricted growth of the prey corrected for the confrontation with itself (on the plate marked with red line, whereas % inhibition is marked with green arrow); conditional color formatting (the gradient from green to red marks the trend from lowest to the highest value) is applied for both panels independently. Aa, Bc and Ss indicate *A. alternata*, *B. cinerea*, and *S. sclerotiorum*, respectively. N means number of strains, av average value, sd standard deviation.

4. Discussion

In this study we extended our earlier findings [19] of *Trichoderma* species diversity in the rhizosphere of *C. arabica* in Ethiopia to coffee endophytes. Despite the potential benefit for *C. arabica*, the community of its endophytic microorganisms has not received much attention. In fact, the only studies so far have been focusing on endophytic bacteria [41], fungal foliar endosymbionts [8] and the presence of ochratoxin producing *Penicillium* spp. [42] in *C. arabica*. In contrast, in this study we focused on the diversity of fungi isolated directly from the healthy coffee roots and on screening for endophytic *Trichoderma* strains that can effectively combat two major pathogens of tracheomycosis, *G. xylarioides*, and FOX. We also tested the general ability of endophytic *Trichoderma* to inhibit further ascomyceteous plant pathogens and compared it to those of the saprophytic *Trichoderma* strains.

4.1. The Narrow Ecological Niche inside Coffee Roots Favors Diverse Community of Endophytic Fungi

C. arabica and other plants rich in xanthine alkaloids such as caffeine represent a special ecological and evolutionary challenge for microorganisms that colonize them. However, the recent studies report on a diversity of fungal endophytes in *Theobroma cacao* (cacao tree, [18]) and *C. arabica* (coffee plant, [8]). The surveys on endophytic fungi in coffee performed in Colombia, Hawaii, and Puerto Rico showed the presence of *Colletotrichum*, *Fusarium*, *Penicillium*, *Xylaria* as the most common genera but also included several entomopathogenic fungi, such as *Acremonium* spp., *Beauveria* spp., *Cladosporium* spp., *Clonostachys* spp., and *Paecilomyces* spp. [8]. However, in this study 63% of fungi isolated from healthy coffee roots represented *Trichoderma* and *Fusarium* species. Thus, our results show that there are different endophytic fungal communities colonizing leaves (see [8]) and roots of *C. arabica*. The fact that *Trichoderma* was isolated from *C. arabica* roots but not from leaves support the suggestion that *Trichoderma* dominates in the rhizosphere community of coffee plants [19], where it plays important role for the plant protection founding special external and also endophytic association with the plant. Understanding endophytic communities is of great importance for improvement of plant health and it is one of the most poorly explored areas in agricultural biotechnology [8].

4.2. Diversity of Endophytic *Trichoderma* in Roots of *C. arabica* is Unique

Only two of 50 *Trichoderma* isolates (both belonging to *T. sp.* C.P.K. 1833) had *tef1* allele which has been found before in about 5000 *Trichoderma* isolates collected world-wide while all others possessed alleles either unique or previously seen in the rhizosphere of *C. arabica* in Ethiopia (see [19]).

The diversity of endophytic *Trichoderma* strains in *C. arabica* was essentially lower compared to the corresponding rhizosphere [19] what indicates that not all *Trichoderma* strains present in the rhizosphere are capable to colonize the roots. For this opportunistic avirulent symbiotic relationship it is crucial *Trichoderma* to penetrate into the plant's root system and persistently survive within living plant tissues [13,43]

Consistent with our results on rhizosphere fungal community in *C. arabica* [19], *T. hamatum* was the most abundant species inhabiting the roots of coffee plant. As expected, *T. hamatum* strains from both ecological niches were sharing the same *tef1* allele, different from any other, also African strains of this species.

Strains belonging to *Harzianum-Catoptron* Clade [37] were abundantly isolated, yet the detailed phylogeny revealed that they are members of three different species. The majority of this strains were attributed to the putative new species *T. sp. C.P.K. 1812*, so far only known by a single strain found by Mulaw *et al.* [19] in the rhizosphere of *C. arabica*. The second numerous group revealed a novel subclade within *H. 'pseudoharzianum'* sensu Druzhinina *et al.* [37] branching closely to the putative new agamospecies *T. sp. 'afroharzianum'* nom. prov. [37]. However, due to the intensive sexual recombination inside this group the taxonomic position of this subclade must be assessed by multiloci phylogeny elsewhere. Further two isolates belonging to the *Harzianum-Catoptron* Clade were attributed to the new cosmopolitan species *T. sp. C.P.K. 1833* (*T. 'ethiopionense'* nom. prov, ms in preparation) abundantly found in rhizosphere of *C. arabica* but also in soils from Siberia [22], in Eastern Europe (R. Labuda, personal communication) and in Central America [26] suggesting the cosmopolitan distribution and saprotrophic nature of this species.

The recently formally described species *T. flagellatum* [39] was first found in our survey and was neither detected in rhizosphere of *C. arabica* nor elsewhere so far.

Many *Trichoderma* strains which were recently isolated as endophytes from tropical *Theobroma cacao* were described as new species; *Trichoderma ovalisporum* [44], *Trichoderma martiale* [16], *Trichoderma stromaticum* [45], *Trichoderma theobromicola*, and *Trichoderma paucisporum* [17], *Trichoderma koningiopsis* [46], and *Trichoderma evansii* [18]. Furthermore, an endophytic *Trichoderma* from Chinese yew (*Taxus mairei*), *T. taxi*, was also recently described [47]. In this survey we isolated a novel endophytic species *T. flagellatum* isolated from healthy roots of *C. arabica*. Its formal description was recently published by Samuels *et al.* [1]. Continuous discovery of species novel to science, which were isolated as endophytes likely resembles the intensive speciation in such narrow ecological niches. Our data also show that the Ethiopian semi-natural coffee soil ecosystem likely underwent major isolation, which was beneficial for formation of not only distinct genotypes but also new species. Mulaw *et al.* [19] emphasised that *C. arabica* rhizosphere in Ethiopia has been a hot spot for speciation of several *Trichoderma* spp. as *C. arabica* is known to display a high genetic diversity in Ethiopia [10] and Tanzania [48], and this fact should gave rise to new *Trichoderma* populations and taxa capable of establishing themselves in the rhizosphere of this genetically highly variable plant. However, due to the accumulation of various alkaloids and other biologically active compounds *C. arabica* may not be an easy target for an opportunistic attack and the adaptation to endophytism might require a longer evolutionary interval.

4.3. *G. xylarioides* is not Present in the Community of Endophytic Fungi from Healthy Coffee Plants

The finding of *Fusarium* spp. as endophytes of healthy *C. arabica* is intriguing; the majority of them belonged to the *G. fujikuroi* and *G. intricans* species complexes. *G. fujikuroi*/*F. solani* is one of the most frequently isolated fungus from soil and plant debris, but it is also a host-specific pathogen of a number of agriculturally important plants, including pea, cucurbits, and sweet potato [28,49,50]. *G. intricans*/*F. equiseti*, in contrast, is considered as a rather weak pathogen, yet capable of colonizing roots and eventually causing disease symptoms in a broad range of crop plants [51]. The *EF-1a* allele of endophytic *G. fujikuroi*/*F. solani* species complex shared highest similarity to isolates from tropical soil assigned as *F. solani* and *F. falciforme*. One of the most similar BLAST hits (DQ247630) even has been

isolated as a parasite of a *Phytium* sp. They likely represent a tropical opportunistic population, which have settled in the rhizosphere of *C. arabica*.

The second agent of coffee tracheomycosis, *G. xylarioides* was not detected in the community of endophytic strains of *C. arabica*. These findings might imply that this agent of the coffee wilt disease has been introduced to Ethiopia, as the occurrence of *G. xylarioides* on *C. arabica* in Ethiopia was first detected in the early 1970s [12], yet this should be confirmed in further investigations. Furthermore, the comparison of *G. xylarioides* isolates from different coffee species, also including earlier isolates, revealed host specialization of this pathogen on *Coffea* spp. [12].

4.4. Endophytic Trichoderma Strains Exhibit High Antifungal Potential

Several epiphytic and endophytic *Trichoderma* species and strains are already being well studied and further recommended as potential biological control agents against phytopathogenic fungi such as *Phytophthora capsici* in hot pepper [52], *Moniliophthora roreri*, *M. fide*, *M. perniciosa*, and *Phytophthora* species in cacao trees [43,53]. *Trichoderma* strains being studied for their potential to control cacao diseases and ameliorate damage caused by abiotic stresses also have an ability to alter cacao gene expression during colonization [43].

Our data revealed high antagonistic potential against the two agents of coffee tracheomycosis, *G. xylarioides* and FOX. However, the endophytic strains less successfully combated FOX, which was also isolated from roots of *C. arabica*, then *G. xylarioides*, which was not detected in roots of healthy plants. This likely reflects a degree of adaptation between endophytic FOX and *Trichoderma* in this specialized native environment.

Furthermore, the endophytic *Trichoderma* strains expressed high mycoparasitic potential against three tested plant pathogens (*A. alternata*, *B. cinerea*, and *S. sclerotiorum*). Their antagonistic activity was in range or even higher with those from saprotrophic strains of *T. hamatum* and *H. 'pseudoharzianum'*. The growth of *Fusarium* pathogens was efficiently inhibited by the endogenous *Trichoderma* strains, therefore these endophytic *Trichoderma* spp. may represent attractive candidates for biocontrol of coffee tracheomycosis and eventually also for other fungal plant diseases. Particularly the putatively exclusive endophytic species (*T. flagellatum* and *T. sp. C.P.K. 1812*) are able to antagonize *G. xylarioides* and FOX which cause coffee tracheomycosis, and additionally also successfully inhibit growth of other plant pathogens. The strain C.P.K. 3340, belonging to the putative new species *T. sp. C.P.K. 1812*, exhibited the strongest antagonistic potential against both *Fusarium* species tested and possessed a great ability to overgrow other three pathogens, thus rendering a potential biocontrol candidate that could simultaneously combat several plant diseases. Herewith, we propose *in vivo* experiments with novel endophytic *Trichoderma* species on the *C. arabica* cultivars to assess effective value of their application in future.

5. Conclusions

In this study we confirmed that endophytic *Trichoderma* spp. from *C. arabica* display antagonistic activity against *G. xylarioides* and FOX, the two *Fusarium* species found in wilted coffee roots. The diversity of *Trichoderma* inside *C. arabica* roots is essentially lower compared to rhizosphere and is composed of mainly endemic species which are either new to science or were previously detected in

rhizosphere of this plant. Endophytic strains of the common opportunistic species *T. hamatum* and *H. 'pseudoharzianum'* possess unique DNA barcodes suggesting emerging genetic isolation. Strains of the putative new species *T. sp. C.P.K. 1812* and recently described *T. flagellatum* so far isolated only from *C. arabica* roots from Ethiopia have shown high mycoparasitic capacity against the studied set of pathogens and therefore may be recommended for the integrated disease management of coffee plants in Ethiopia.

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Conflicts of Interest

The authors declare no conflict of interest.

References

1. Bills, G.F. Isolation and Analysis of Endophytic Fungal Communities from Woody Plants. In *Systematics, Ecology and Evolution of Endophytic Fungi in Grasses and Woody Plants*; Redlin, S., Carris, L.M., Eds.; APS Press: St. Paul, MN, USA, 1996; pp. 31–65.
2. Larran, S.; Perello, A.; Simon, M.R.; Moreno, V. Isolation and analysis of endophytic microorganisms in wheat (*Triticum aestivum* L.) leaves. *World J. Microb. Biot.* **2002**, *18*, 683–686.
3. Redlin, S.C.; Carris, L.M. Endophytic Fungi in Grasses and Woody Plants. In *Systematics, Ecology and Evolution of Endophytic Fungi in Grasses and Woody Plants*; Redlin, S., Carris, L.M., Eds.; APS press: St. Paul, MN, USA, 1996; p. 216.
4. Marshall, D.; Tunali, B.; Nelson, L.R. Occurrence of fungal endophytes in species of wild *Triticum*. *Crop Sci.* **1996**, *39*, 1507–1512.
5. Huang, Y.; Jianfeng, W.G.L.; Zhonghui, Z.; Wenjin, S. Anti tumor and antifungalactivities in endophytic fungi isolated from pharmaceutical plants *Taxus mairei*, *Cephalataxus fortunei* and *Torreya grandis*. *FEMS Immunol. Med. Microbiol.* **2001**, *31*, 163–167.
6. Arnold, A.E.; Mejía, L.C.; Kylo, D.; Rojas, E.I.; Maynard, Z.; Robbins, N.; Herre, E.A. Fungal endophytes limit pathogen damage in a tropical tree. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 15649–15654.
7. Niere, B.; Gold, C.S.; Coyne, D. Can fungal endophytes control soilborne pests in banana? *Bull. OILB/SROP* **2004**, *27*, 203–209.
8. Santamaría, J.; Bayman, P. Fungal epiphytes and endophytes of Coffee Leaves (*Coffea arabica*). *Microb. Ecol.* **2005**, *50*, 1–8.
9. Sylvain, P. Ethiopian coffee—its significance to world coffee problems. *Econ. Bot.* **1958**, *12*, 111–139.

10. Aga, E.; Bryngelsson, T.; Bekele, E.; Salomon, B. Genetic diversity of forest arabica coffee (*Coffea arabica* L.) in Ethiopia as revealed by random amplified polymorphic DNA (RAPD) analysis. *Hereditas* **2003**, *138*, 36–46.
11. Geiser, D.M.; Ivey, M.L.L.; Hakiza, G.; Juba, J.H.; Miller, S.A. *Gibberella xylarioides* (anamorph: *Fusarium xylarioides*), a causative agent of coffee wilt disease in Africa, is a previously unrecognized member of the *G. fujikuroi* species complex. *Mycologia* **2005**, *97*, 191–201.
12. Girma, A.; Hulluka, M.; Hindorf, H. Incidence of tracheomycosis, *Gibberella xylarioides* (*Fusarium xylarioides*), on Arabica coffee in Ethiopia. *Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz* **2001**, *108*, 136–142.
13. Harman, G.E.; Howell, C.R.; Viterbo, A.; Chet, I.; Lorito, M. *Trichoderma* species: Opportunistic avirulent plant symbionts. *Nat. Rev. Microbiol.* **2004**, *2*, 43–56.
14. Druzhinina, I.S.; Seidl-Seiboth, V.; Herrera-Estrella, A.; Horwitz, B.A.; Kenerley, C.M.; Monte, E.; Mukherjee, P.K.; Zeilinger, S.; Grigoriev, I.V.; Kubicek, C.P. *Trichoderma*: The genomics of opportunistic success. *Nat. Rev. Microbiol.* **2011**, *9*, 749–759.
15. Zhang, C.L.; Druzhinina, I.S.; Kubicek, C.P.; Xu, T. Biodiversity of *Trichoderma* in China: Evidence for a North to South difference of species distribution in East Asia. *FEMS Microbiol. Letts.* **2005**, *251*, 251–257.
16. Hanada, R.E.; de Jorge Souza, T.; Pomella, A.W.; Hebbbar, K.P.; Pereira, J.O.; Ismaiel, A.; Samuels, G.J. *Trichoderma martiale* sp. nov., a new endophyte from sapwood of *Theobroma cacao* with a potential for biological control. *Mycol. Res.* **2008**, *112*, 1335–1343.
17. Samuels, G.J.; Suarez, C.; Solis, K.; Holmes, K.A.; Thomas, S.E.; Ismaiel, A.; Evans, H.C. *Trichoderma theobromicola* and *T. paucisporum*: Two new species isolated from cacao in South America. *Mycol. Res.* **2006**, *110*, 381–392.
18. Samuels, G.J.; Ismaiel, A. *Trichoderma evansii* and *T. lieckfeldtia*: Two new *T. hamatum*-like species. *Mycologia* **2009**, *101*, 142–156.
19. Mulaw, T.B.; Kubicek, C.P.; Druzhinina, I.S. The distinguished diversity of *Trichoderma* is associated with rhizosphere of *Coffea arabica* in highland forests of Ethiopia. *Diversity* **2010**, *2*, 527–549.
20. Mysore, V.T.; Basavanna, M.; Monnanda, S.N.; Harishchandra, S.P.; Kukkundoor, R.K.; Ven, S.; Hunthrike, S.S. Endophytic fungal assemblages from inner and twig of *Terminalia arjuna* W. and A. (*Combretaceae*). *World J. Microbiol. Biotechnol.* **2005**, *21*, 1535–1540.
21. White, T.J.; Bruns, T.; Lee, S.; Taylor, J. Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In *PCR Protocols: A Guide to Methods and Applications*; Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J., Eds.; Academic Press: San Diego, CA, USA, 1990; pp. 315–322.
22. Kullnig-Gradinger, C.M.; Szakacs, G.; Kubicek, C.P. Phylogeny and evolution of the fungal genus *Trichoderma*—a multigene approach. *Mycol. Res.* **2002**, *106*, 757–767.
23. Chaverri, P.; Castlebury, L.A.; Samuels, G.J.; Geiser, D. Multilocus phylogenetic structure within the *Trichoderma/Hypocrea lixii* complex. *Mol. Phylogenet. Evol.* **2003**, *27*, 302–313.

24. Druzhinina, I.S.; Komoń-Zelazowska, M.; Kredics, L.; Hatvani, L.; Antal, Z.; Belayneh, T.; Kubicek, C.P. Alternative reproductive strategies of *Hypocrea orientalis* and genetically close but clonal *Trichoderma longibrachiatum*, both capable of causing invasive mycoses of humans. *Microbiology* **2008**, *154*, 3447–3459.
25. Altschul, S.F.; Gish, W.; Miller, W.; Myers, E.W.; Lipman, D.J. Basic local alignment search tool. *J. Mol. Biol.* **1990**, *215*, 403–410.
26. Druzhinina, S.I.; Kopchinskiy, G.A.; Komon, M.; Bissett, J.; Szakacs, G.; Kubicek, P.C. An oligonucleotide barcode for species identification in *Trichoderma* and *Hypocrea*. *Fungal Genet. Biol.* **2005**, *42*, 813–828.
27. Kopchinskiy, A.; Komon, M.; Kubicek, C.P.; Druzhinina, I.S. TRICHOBLAST: A multilocus database for *Trichoderma* and *Hypocrea* identifications. *Mycol. Res.* **2005**, *109*, 658–660.
28. O'Donnell, K. Molecular phylogeny of the *Nectria haematococca-Fusarium solani* species complex. *Mycologia* **2000**, *92*, 919–938.
29. Geiser, D.M.; Jiménez-Gasco, M.; Kang, S.; Makalowska, I.; Veeraraghavan, N.; Ward, T.J.; Zhang, N.; Kulda, G.A.; O'Donnell, K. FUSARIUM-ID v.1.0: A DNA sequence database for identifying *Fusarium*. *Eur. J. Plant Pathol.* **2004**, *110*, 473–479.
30. Thompson, J.D.; Gibson, T.J.; Plewniak, F.; Jeanmougin, F.; Higgins, D.G. The ClustalX windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **1997**, *25*, 4876–4882.
31. Nicholas, K.B.; Nicholas, H.B., Jr.; Deerfield, D.W., II. GeneDoc: Analysis and Visualization of Genetic Variation, EMBNEW.NEWS 1997, 4:14. Available online: <http://www.psc.edu/biomed/genedoc> (accessed on 28 August 2013).
32. Ronquist, F.; Huelsenbeck, J.P. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **2003**, *19*, 1572–1574.
33. Tavaré S. Some Probabilistic and Statistical Problems in the Analysis of DNA Sequences. In *Some Mathematical Questions in Biology—DNA Sequence Analysis*; Miura, R.M., Ed.; American Mathematical Society: Providence, RI, USA, 1986; pp. 57–86.
34. Atanasova, L.; Jaklitsch, W.M.; Komon-Zelazowska, M.; Kubicek, C.P.; Druzhinina, I.S. Clonal species *Trichoderma parareesei* sp. nov. likely resembles the ancestor of the cellulase producer *Hypocrea jecorina*/*T. reesei*. *Appl. Environ. Microbiol.* **2010**, *76*, 7259–7267.
35. Leaché A.D.; Reeder, T.W. Molecular systematics of the Eastern Fence lizard (*Sceloporus undulatus*): A comparison of parsimony, likelihood and Bayesian approaches. *Syst. Biol.* **2002**, *51*, 44–68.
36. Ortiz, A.; Orduz, S. In vitro evaluation of *Trichoderma* and *Gliocladium* antagonism against the symbiotic fungus of the leaf-cutting ant *Atta cephalotes*. *Mycopathologia* **2001**, *150*, 53–60.
37. Druzhinina, I.S.; Kubicek, C.P.; Komon-Zelazowska, M.; Mulaw, T.B.; Bissett, J. The *Trichoderma harzianum* demon: Complex speciation history resulting in coexistence of hypothetical biological species, recent agamospecies and numerous relict lineages. *BMC Evol. Biol.* **2010**, doi:10.1186/1471-2148-10-94.
38. Bisset, J.; Szakacs, G.; Nolan, C.A.; Druzhinina, S.I.; Gradinger, C.M.; Kubicek, C.P. New species of *Trichoderma* from Asia. *Can. J. Bot.* **2003**, *81*, 570–586.

39. Samuels, G.J.; Ismaiel, A.; Mulaw, T.B.; Szakacs, G.; Druzhinina, I.S.; Kubicek, C.P.; Jaklitsch, W.M. The *Longibrachiatum* Clade of *Trichoderma*: A revision with new species. *Fungal Divers.* **2012**, *55*, 77–108.
40. Druzhinina, I.S.; Komoń-Zelazowska, M.; Ismaiel, A.; Jaklitsch, W.; Mullaw, T.; Samuels, G.J.; Kubicek, C.P. Molecular phylogeny and species delimitation in the section *Longibrachiatum* of *Trichoderma*. *Fungal Genet. Biol.* **2012**, *49*, 358–368.
41. Vega, F.E.; Pava-Ripoll, M.; Posada, F.; Buyer, J.S. Endophytic bacteria in *Coffea arabica* L. *J. Basic Microbiol.* **2005**, *45*, 371–380.
42. Vega, F.E.; Posada, F.; Peterson, S.W.; Gianfagna, T.J.; Chaves, F. *Penicillium* species endophytic in coffee plants and ochratoxin A production. *Mycologia* **2006**, *98*, 31–42.
43. Bailey, B.A.; Strem, M.D.; Wood, D. *Trichoderma* species form endophytic associations within *Theobroma cacao* trichomes. *Mycol. Res.* **2009**, *113*, 1365–1376.
44. Holmes, K.A.; Schroers, H.-J.; Thomas, S.E.; Evans, H.C.; Samuels, G.J. Taxonomy and biocontrol potential of a new species of *Trichoderma* from the Amazon basin of South America. *Mycol. Prog.* **2004**, *3*, 199–210.
45. Samuels, G.J.; Pardo-Schultheiss, R.; Hebbar, K.P.; Lumsden, R.D.; Bastos, C.N.; Costa, J.C.; Bezerra, J.L. *Trichoderma stromaticum* sp. nov., a parasite of the cacao witches broom pathogen. *Mycol. Res.* **2000**, *104*, 760–764.
46. Samuels, G.J.; Dodd, S.L.; Lu, B.-S.; Petrini, O.; Schroers, H.-J.; Druzhinina, I.S. The *Trichoderma koningii* aggregate species. *Stud. Mycol.* **2006**, *56*, 67–133.
47. Zhang, C.L.; Liu, S.P.; Lin, F.C.; Kubicek, C.P.; Druzhinina, I.S. *Trichoderma taxi* sp. nov., an endophytic fungus from Chinese yew *Taxus mairei*. *FEMS Microbiol. Lett.* **2007**, *270*, 90–96.
48. Masumbuko, L.I.; Bryngelsson, T.; Mneney, E.E.; Salomon, B. Genetic diversity in Tanzanian Arabica coffee using random amplified polymorphic DNA (RAPD) markers. *Hereditas* **2003**, *139*, 56–63.
49. Booth, C. *The Genus Fusarium*; Commonwealth Mycological Institute: Surrey, UK, 1971.
50. Zhang, N.; O'Donnell, K.; Sutton, D.A.; Nalim, F.A.; Summerbell, R.C.; Padhye, A.A.; Geiser, D.M. Members of the *Fusarium solani* species complex that cause infections in both humans and plants are common in the environment. *J. Clin. Microbiol.* **2006**, *44*, 2186–2190.
51. Goswami, R.S.; Dong, Y.; Punja, Z.K. Host range and mycotoxin production by *Fusarium equiseti* isolates originating from Ginseng fields. *Can. J. Plant. Pathol.* **2008**, *30*, 155–160.
52. Bae, H.; Sicher, R.C.; Kim, M.S.; Kim, S.H.; Strem, M.D.; Melnick, R.L.; Bailey, B.A. The beneficial endophyte *Trichoderma hamatum* isolate DIS 219b promotes growth and delays the onset of the drought response in *Theobroma cacao*. *J. Exp. Bot.* **2009**, *60*, 3279–3295.
53. Bae, H.; Roberts, D.P.; Lim, H.S.; Strem, M.D.; Park, S.C.; Ryu, C.M.; Melnick, R.L.; Bailey, B.A. Endophytic *Trichoderma* isolates from tropical environments delay disease onset and induce resistance against *Phytophthora capsici* in hot pepper using multiple mechanisms. *Mol. Plant Microbe Interact.* **2011**, *24*, 336–351.