Fungal Diversity (2013) 60:137–151
DOI 10.1007/s13225-012-0216-3

Endophytic fungi harbored in *Cannabis sativa* L.: diversity and potential as biocontrol agents against host plant-specific phytopathogens

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Received: 20 September 2012 / Accepted: 9 November 2012 / Published online: 19 November 2012

Abstract The objective of the present work was isolation, phylogenetic characterization, and assessment of biocontrol potential of endophytic fungi harbored in various tissues (leaves, twigs, and apical and lateral buds) of the medicinal plant, *Cannabis sativa* L. A total of 30 different fungal endophytes were isolated from all the plant tissues which were authenticated by molecular identification based on rDNA ITS sequence analysis (ITS1, 5.8S and ITS2 regions). The Menhinick's index revealed that the buds were immensely rich in fungal species, and Camargo's index showed the highest tissue-specific fungal dominance for the twigs. The most dominant species was *Penicillium copticola* that could be isolated from the twigs, leaves, and apical and lateral buds. A detailed calculation of Fisher's log series index, Shannon diversity index, Simpson's index, Simpson's diversity index, and Margalef's richness revealed moderate overall biodiversity of *C. sativa* endophytes distributed among its tissues. The fungal endophytes were challenged by two host phytopathogens, *Botrytis cinerea* and *Trichothecium roseum*, devising a dual culture antagonistic assay on five different media. We observed 11 distinct types of pathogen inhibition encompassing a variable degree of antagonism (%) on changing the media. This revealed the potential chemodiversity of the isolated fungal endophytes not only as promising resources of biocontrol agents against the known and emerging phytopathogens of *Cannabis* plants, but also as sustainable resources of biologically active and defensive secondary metabolites.

Keywords *Cannabis sativa* · Endophytic fungi · Fungal diversity · Antagonism · *Botrytis cinerea* · *Trichothecium roseum*

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Introduction

Cannabis sativa L. (Cannabaceae) is an annual herbaceous plant, native mainly to Central Asia, that has been in use all over the planet either in the form of narcotic or medicinal preparations or as a source of food and fiber (Jiang et al. 2006). The secondary metabolites of this plant constitute more than 400 compounds (Turner et al. 1980), with the most emphasis being led on cannabinoids. More than 108 cannabinoids have already been discovered (Ahmed et al. 2008; ElSohly and Slade 2005; Fischedick et al. 2010; Hazekamp et al. 2004, 2005; Radwan et al. 2008). Although *Cannabis* is regarded as mainly a drug of abuse at present, cannabinoids are known to have important therapeutic effects such as analgesic, anti-spasmodic, anti-tremor, anti-inflammatory, antioxidant, neuro-protective, and appetite stimulant (Baker et al. 2003; Gomes et al. 2008; Mojzisova and Mojzis 2008; Williamson and Evans 2000). Such pronounced efficacies of cannabinoids have led to the development of various *Cannabis*-based medicines, namely dronabinol (Marinol®, Solvay Pharmaceuticals, Belgium), Sativex (GW Pharmaceuticals, UK), and nabilone (Cesamet®, Valeant Pharmaceuticals International, USA). Although Δ9-tetrahydrocannabinol (Δ9-THC) is considered to be the major psychoactive compound (Pertwee 2006; Sirikantaramas et al. 2005; Taura et al. 1995), there is still a lot of intensive investigation to verify if pure cannabinoids provide better therapeutic effect over the whole plant extracts,

and the worth of other compounds in *Cannabis*-based medicinal use (ElSohly et al. 2003; Grotenhermen and Müller-Vahl 2012; Russo and McPartland 2003; Wachtel et al. 2002; Williamson and Evans 2000).

C. sativa is commonly known as 'hemp'. Owing to the potent phytochemical constituents and diverse use of this plant by humans, an overall fallacy that "hemp has no enemies" (Dewey 1914) has developed. Unfortunately, this plant is attacked by a plethora of phytopathogens leading to a number of diseases (McPartland 1996) prevalent in every organ (such as leaf, flower, stem and root) and growth stage (seedling to mature plant). A number of specific and non-specific bacteria and fungi have been found to be associated with the plant as pathogens, and responsible for different stress symptoms and diseases (Grotenhermen and Müller-Vahl 2012; Kurup et al. 1983; McPartland 1983, 1994; Schwartz 1985; Taylor et al. 1982). In particular, more than 80 different fungal species have been discovered so far that poses some form of threat to *Cannabis* plants (Hockey 1927; McPartland 1995). However, two of the most threatening diseases of *C. sativa* have been shown to be caused by the phytopathogens *Botrytis cinerea* and *Trichothecium roseum* (McPartland 1996). On the one hand, *B. cinerea* attacks the leaves, flowers, stems and branches of this plant leading to the disease known as 'gray mold', which can completely destroy the plant within 1 week (Barloy and Pelhate 1962). This fungal pathogen forms a grey brown mat and encircles leaves, stems and flowers and can even spread epidemic disasters in the field (van der Werf and van Geel 1994; van der Werf et al. 1995). *B. cinerea* also causes another disease called 'damping off' where it weakens the seeds or seedlings before or after they germinate, or even kill the seedlings (Bush Doctor 1985). On the other hand, *T. roseum* attacks the leaves and flowers of *C. sativa* plants causing the dreaded 'pink rot' disease, which is a greenhouse threat for cultivars (McPartland 1991). Although some sporadic attempts have been made for the elimination of the fungal pathogens from this plant (Ungerlerder et al. 1982; Kurup et al. 1983; Levitz and Diamond 1991; Bush Doctor 1993), a more comprehensive, practical and ecologically relevant means to eradicate the pathogen-mediated diseases in *Cannabis* is necessary. It is, thus, highly desirable to effectively address these threats to prevent the loss of these medicinally relevant plants and drastically reduce the amount of hazards caused by these specific and/or other opportunistic pathogens.

Plant associated bacterial and fungal communities play an important role in balancing the ecosystem. Endophytic microorganisms ('endophytes') are a group of highly assorted organisms that internally infect living plant tissues without instigating any noticeable symptom of infection or visible manifestation of disease, and live in mutualistic association with plants for at least a part of their life cycle (Botella and Diez 2011; Hyde and Soytong 2008; Kusari and Spiteller 2012; Kusari et al. 2012b; Purahong and Hyde 2011; Vesterlund et al. 2011). Endophytes, mainly represented by fungi but also by bacteria, have great promise with diverse potential for exploitation (Li et al. 2012; Staniek et al. 2008). A plethora of competent endophytic fungi have already been discovered that are capable of providing different forms of fitness benefits to their associated host plants (Hamilton et al. 2012; Hamilton and Bauerle 2012). For example, these organisms have demonstrated the capacity to produce a diverse range of biologically active secondary metabolites (Aly et al. 2010; Debbab et al. 2012; Gunatilaka 2006; Kharwar et al. 2011; Staniek et al. 2008; Strobel and Daisy 2003; Strobel et al. 2004; Suryanarayanana et al. 2009; Zhang et al. 2006), occasionally including those similar to their associated host plants (Eyberger et al. 2006; Kusari et al. 2008, 2009a, b, c, 2011, 2012a), and induce host plant tolerance to environmental stress, herbivory, heat, salt, disease and drought (Arnold et al. 2003; Márquez et al. 2007; Porras-Alfaro and Bayman 2011; Redman et al. 2002; Rodriguez et al. 2004, 2008; Rodriguez and Redman 2008; Stone et al. 2000; Waller et al. 2005).

The objective of the work reported in this manuscript was to evaluate the diversity of endophytic fungi isolated from different tissues of *Cannabis sativa* L., and further screen them as potential biocontrol agents against two major fungal pathogens of the plant, namely *Botrytis cinerea* and *Trichothecium roseum*. Based on the knowledge that the biosyntheses of secondary metabolites in endophytes are dependent on the culture parameters and available nutrition (OSMAC, One Strain MAny Compounds) (Bode et al. 2002), we further evaluated the antagonistic effects of isolated endophytes against the two host-specific pathogens on five different media. To the best of our knowledge, this is the first report of the incidence, diversity, phylogeny, and assessment of biocontrol potential of endophytic fungi harbored in *C. sativa*.

Materials and methods

Collection, identification, and authentication of plant material

As part of an effort to identify endophytic fungi that provide fitness benefits to their host plants, *Cannabis sativa* plants were sampled from the Bedrocan BV Medicinal Cannabis (the Netherlands). The plants were identified and authenticated as *C. sativa* by experienced botanists at the Bedrocan BV. Plants specimens are under deposit at Bedrocan BV with voucher numbers (A1)05.41.050710. These plants were then transported to the TU Dortmund, Germany immediately, and processed within 6 h of collection. Import of the plant material was allowed according to the permission of the Federal Institute for Drugs and Medical Devices

(*Bundesinstituts für Arzneimittel und Medizinprodukte*, BfArM), Bonn, Germany under the license number 458 49 89. Different parts of the plants such as fresh leaves, twigs, and apical and lateral buds were carefully excised from the live host plant (roots were unavailable due to legislative restrictions). The excised tissues were washed thoroughly in running tap water followed by deionized (DI) water to remove any dirt sticking to them and stored at 4 °C until the isolation procedure of endophytic fungi was commenced (≤10 min).

Isolation of endophytic fungi and establishment of in vitro axenic cultures

The surface sterilization and isolation of endophytes was done following previously established procedures (Kusari et al. 2009a). The explants were thoroughly washed in running tap water, and small fragments of leaves, twigs, and buds of approximately 10 mm (length) by 5 mm (breadth) were cut with the aid of a flame-sterilized razor blade (same number of fragments for each tissue type). Then, the small tissue fragments were surface-sterilized by sequential immersion in 70 % ethanol for 1 min, 1.3 M sodium hypochlorite (3–5 % available chlorine) for 3 min, and 70 % ethanol for 30 s. Finally, these surface-sterilized tissue pieces were rinsed thoroughly in sterile, double-distilled water for a couple of minutes, to remove excess surface sterilants. The excess moisture was blotted on a sterile filter paper. The surface-sterilized tissue fragments, thus obtained, were evenly placed (four fragments in each plate) in petri dishes (Diagonal GmbH & Co. KG, Germany) containing water agar (WA) medium (Roth, cat. no. 5210.2) amended with streptomycin (100 mg L^{-1}) to eliminate any bacterial growth. The petri dishes were sealed using Parafilm (Diagonal GmbH & Co. KG, Germany). The petri dishes were incubated at 28± 2 °C until fungal growth started. To ensure proper surface sterilization and isolation of fungal endophytes, unsterilized tissue fragments (only washed thoroughly in water) were prepared simultaneously, placed in both WA and Sabouraud agar (SA; Roth, cat. no. X932), and incubated under the same conditions in parallel, to isolate the surface-contaminating fungi (differentiated morphologically by both macroscopic and microscopic evaluation) (Kusari et al. 2009b). The cultures were monitored every day to check the growth of endophytic fungi. The endophytic organisms, which grew out from the sample segments over 4–6 weeks were isolated and subcultured onto a rich mycological medium, SA, and brought into pure culture. To ensure proper surface sterilization, surface-sterilized tissue fragments were imprinted simultaneously in WA as well as SA and incubated under the same conditions in parallel (secondary protocol, 'imprint technique') (Schulz et al. 1998; Sánchez Márquez et al. 2007).

Maintenance and storage of the axenic endophytic fungal isolates

The axenic cultures, obtained above, were coded according to their host tissue origin (L1, L2, etc. from leaves, T1, T2, T3, etc. from twigs, and A1, A2, A3, etc. from apical/lateral buds), and were routinely maintained on PDA, SA and CDA (Czapek-Dox Agar; Merck, Darmstadt, Germany) in active form. For long-term storage, the colonies were preserved in the form of spores (those which readily sporulated in axenic cultures) as well as vegetative form in 15 % (v/v) glycerol at −70 °C. Agar blocks impregnated with mycelia were used directly for storage of the vegetative forms. For the isolation of the genomic DNA of the endophytes, a set of conical flasks of 500 mL capacity each with 100 ml Sabouraud broth (SB; Roth, cat. no. AE23.1) was used with proper autoclaving. The endophytic fungi were inoculated in the respective flasks from the parent axenic cultures. The flasks were incubated at 28±2 °C with proper shaking (150 rev min^{-1}) on a rotary shaker (Heidolph UNIMAX 2010, Germany) over 4–6 weeks.

Total genomic DNA extraction, PCR amplification and sequencing

The total genomic DNA (gDNA) was extracted from the in vitro cultures using peqGOLD fungal DNA mini kit (Peqlab Biotechnologie GmbH, Germany, cat. no. 12-3490-02) strictly following the manufacturer's guidelines. The DNA was then subjected to PCR amplification using primers ITS4 and ITS5 according to White et al. (1990). The amplified fragment consisted of ITS1, 5.8S and ITS2 regions of the rDNA. The PCR reaction was performed in 50 μL reaction mixture containing 10 μL Phusion HF buffer (5X), 1 μL dNTPs (10 mM), 0.5 μL forward primer (100 μM), 0.5 μL reverse primer (100 μM), 3 μL of template DNA, 1 μL of Phusion polymerase (2 U μL^{-1}), and 34 μL of sterile double-distilled water. The PCR cycling protocol consisted of an initial denaturation at 98 °C for 3 min, 30 cycles of denaturation, annealing and elongation at 98 °C for 10 s, 58 °C for 30 s and 72 °C for 45 s. This was followed by a final elongation step of 72 °C for 10 min. As a negative control, the template DNA was replaced by sterile double-distilled water. The PCR amplified products were checked by gel electrophoresis spanning approximately 500–600 bp (base pairs). The PCR products were further purified using peqGOLD micro spin cycle pure kit (Peqlab, cat. no. 12-6293-01) according to the manufacturer's instructions. The amplified products were then sequenced on ABI 3730xl DNA analyzer at GATC Biotech (Cologne, Germany).

Identification of endophytic fungi and phylogenetic evaluation

For strain identification, the sequences were matched against the nucleotide database using the Basic Local Alignment Search Tool (BLASTn) of the US National Centre for Biotechnology Information (NCBI) for the final identification of the endophytes. The sequences were aligned using ClustalW-Pairwise Sequence Alignment of the EMBL Nucleotide Sequence Database. The sequence alignments were trimmed and verified by the MUSCLE (UPGMA) algorithm (Edgar 2004) using MEGA5 software (Tamura et al. 2011). When the similarity between a particular problem-sequence and a phylogenetically associated reference-sequence was ≥99 %, only then the sequences were considered to be conspecific (Yuan et al. 2010). The phylogenetic tree was reconstructed and the evolutionary history inferred using the Neighbor-Joining method (Saitou and Nei 1987). The robustness of the internal branches was also assessed with 1000 bootstrap replications (Felsenstein 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and were calculated in the units of the number of base substitutions per site. The sequences of this study were deposited at the EMBL-Bank. The accession numbers are detailed in Table 1.

Table 1 Summary of the fungal endophytes isolated from various tissues of *C. sativa* with their respective strain codes, EMBL-Bank accession numbers, and closest affiliations of the representative isolates in the GenBank according to rDNA ITS analysis

Strain number (endophyte)	Part (tissue) of the plant	EMBL-Bank accession number	Most closely related strain (accession number)	Reference	Maximum identity (%)
L1	Leaf	HE962579	*Penicillium copticola* (JN617685.1)	Houbraken et al. 2011	98
L2		HE962580	*Penicillium copticola* (JN617685.1)	Houbraken et al. 2011	99
L3		HE962581	*Penicillium copticola* (JN617685.1)	Houbraken et al. 2011	99
L4		HE962582	*Penicillium copticola* (JN617685.1)	Houbraken et al. 2011	99
L5		HE962482	*Chaetomium globosum* (HQ914911.1)	NA	99
L6		HE962576	*Chaetomium globosum* (JF773585.1)	NA	99
L7		HE962577	*Eupenicillium rubidurum* (HQ608058.1)	Rodrigues et al. 2011	99
L8		HE962578	*Eupenicillium rubidurum* (HQ608058.1)	Rodrigues et al. 2011	99
T1	Twig	HE962583	*Penicillium copticola* (JN617685.1)	Houbraken et al. 2011	99
T2		HE962584	*Penicillium copticola* (JN617685.1)	Houbraken et al. 2011	99
T3		HE962585	*Penicillium copticola* (JN617685.1)	Houbraken et al. 2011	99
T4		HE962586	*Penicillium copticola* (JN617685.1)	Houbraken et al. 2011	98
T5		HE962587	*Penicillium copticola* (JN617685.1)	Houbraken et al. 2011	99
T6		HE962588	*Penicillium sp.* (JF439496.1)	Han et al. 2011	99
A1	Apical/lateral buds	HE962589	*Penicillium copticola* (JN617685.1)	Houbraken et al. 2011	99
A2		HE962590	*Penicillium copticola* (JN617685.1)	Houbraken et al. 2011	99
A3		HE962591	*Penicillium copticola* (JN617685.1)	Houbraken et al. 2011	99
A4		HE962592	*Paecilomyces lilacinus* (GU980015.1) [syn. *Purpureocillium lilacinum*]	NA	99
A5		HE962593	*Penicillium copticola* (JN617685.1)	Houbraken et al. 2011	99
A6		HE962594	*Penicillium sumatrense* (AY213677.1)	Rakeman et al. 2005	99
A7		HE962595	*Penicillium meleagrinum var. viridiflavum* (HM469412.1)	Jang et al. 2011	99
A8		HE962596	*Penicillium copticola* (JN617685.1)	Houbraken et al. 2011	99
A9		HE962597	*Penicillium copticola* (JN617685.1)	Houbraken et al. 2011	99
A10		HE962598	*Penicillium copticola* (JN617685.1)	Houbraken et al. 2011	99
A11		HE962599	*Penicillium copticola* (JN617685.1)	Houbraken et al. 2011	99
A12		HE962600	*Aspergillus versicolor* (FJ878627.1)	Arabatzis et al. 2011	99
A13		HE962601	*Penicillium copticola* (JN617685.1)	Houbraken et al. 2011	99
A14		HE962602	*Penicillium copticola* (JN617685.1)	Houbraken et al. 2011	99
A15		HE962603	*Penicillium sumatrense* (AY213677.1)	Rakeman et al. 2005	99
A16		HE962604	*Penicillium copticola* (JN617685.1)	Houbraken et al. 2011	99

NA not available (not published or not yet published)

Evaluation and quantification of fungal diversity

Species richness among the isolated endophytic fungi was determined by calculating the Menhinick's index (D_{mn}) (Whittaker 1977) using the following equation:

$$D_{mn} = \frac{s}{\sqrt{N}}$$

Therein, s is the number of different endophytic species in a sample (in this case, plant tissue) and N is the total number of isolated endophytic fungi in a given sample.

The fungal dominance was then determined by Camargo's index ($1/D_{mn}$), where D_{mn} represents species richness. A species was defined as dominant if $P_i > 1/D_{mn}$ (Camargo 1992), where P_i is the relative abundance of a species, i defined as the number of competing species present in the community. The species diversity was also evaluated comparing the whole community of isolated endophytic fungi from all tissues of the plant to understand whether these organisms were distributed randomly through the tissues, aggregated, or uniformly distributed (Lambshead and Hodda 1994). Furthermore, to quantify the endophytic fungal diversity of *C. sativa* in different tissues, Fisher's log series index (α), the Shannon diversity index (H'), Simpson's index (D) and Simpson's diversity index (1-D), and Margalef's richness (D_{mg}) were calculated (Fisher et al. 1943; Hoffman et al. 2008; Lambshead et al. 1983; Simpson 1949; Suryanarayanan and Kumaresan 2000; Margalef 1958; Tao et al. 2008) using the following equations, respectively:

$$\alpha = \frac{N(1-x)}{x}$$

Where, x was calculated by

$$\frac{S}{N} = \frac{(1-x)}{x} ln \frac{1}{(1-x)}$$

$$H' = -\sum_i P_i \; ln(P_i)$$

Where, H' values could start from 0 (only one species present with no uncertainty as to what species each individual will be) and go higher revealing high uncertainty as species are relatively evenly distributed.

$$D = \sum_i \frac{n_i(n_i-1)}{N(N-1)}$$

Where, D could range between 0 (infinite diversity) and 1 (no diversity).

$$D_{mg} = \frac{(S-1)}{ln(N)}$$

Therein, N is the number of individuals (defined by numbers of endophytic fungal isolates), S is the number of taxa (ITS genotype), n is the total number of endophytic microorganisms of a particular species, and i is the proportion of species relative to the total number of species (P_i). Taxon accumulation curves and bootstrap estimates of total species richness based on recovered fungal isolates were generated using the software BioDiversity Pro (McAleece et al. 1997).

Pathogens used for antagonistic assays

The endophytic fungi were tested against the known pathogens of the *Cannabis* plant, which were obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. The fungi *Botrytis cinerea* (accession number DSM 5145) and *Trichothecium roseum* (accession number DSM 63066) were employed. The medium used for the activation of the microorganisms were malt extract agar (MEA; Roth, cat. no. X923.1) and potato dextrose agar (PDA; Roth, cat. no. X931.1). Activation was performed strictly according to the DSMZ guidelines. The activated strains were routinely maintained on PDA, MEA, and SA respectively. All procedures were carried out under aseptic conditions.

In vitro antagonistic activity of endophytes against host phytopathogens

The in vitro antagonistic behavior of all endophytes was tested against the host plant-specific pathogens *B. cinerea* and *T. roseum* using the dual culture plate antagonism assay method established earlier (Chamberlain and Crawford 1999; Miles et al. 2012; Trejo-Estrada et al. 1998), suitably modified. Five different kinds of media were used for the bioassay namely SA, MEA, PDA, WA and Nutrient agar (NA; Difco, cat. no. 234000) respectively. The plates were prepared in 90 mm sterile petri dishes (Diagonal GmbH & Co. KG, Germany) with approximately 22 mL of the media, yielding a final depth of 4 mm. Then, 5 mm plugs of each endophyte and pathogen were co-cultured in the five different media mentioned above and incubated at 28±2 °C. The plugs were placed at the two opposite edge of the petri dishes facing each other. The pathogens alone were inoculated as controls. The diameter of growth of both endophyte and pathogen were monitored daily and recorded at 5, 10 and 15 days, respectively. All control and test plates were run in duplicates. Relative growth inhibitions (% antagonism) were calculated against the control plates for each of the

endophyte-pathogen combinations, in each of the five medium used in the bioassay. Percentage antagonism was calculated by using a modified equation mentioned below (Chamberlain and Crawford 1999):

$$\textit{Radial growth of pathogen in presence of endophyte}(RG) = \frac{\textit{total growth of pathogen} - \textit{fungal plug inoculum of pathogen}}{2}$$

$$\textit{Radial growth of pathogen in absence of endophyte}(\textit{control}) = \frac{\textit{total growth of pathogen in control plate} - \textit{fungal plug inoculum of pathogen}}{2}$$

$$\%Antagonism = 1 - \frac{RG}{control} \times 100$$

Results

Identification and characterization of the endophytic fungi

A plethora of fungal endophytes were isolated from the various tissues of *C. sativa* L. such as leaves, twigs, and apical and lateral buds. A total of 30 endophytic fungal isolates were isolated from various tissues, whereby the buds hosted the largest number of endophytes (16 isolates) followed by the leaves (8 isolates) and finally the twigs (6 isolates) (Table 1). The selective media supporting the pure culture of fungi was noted, and the isolates were preserved in our microbial library. The endophytic fungi were authenticated by molecular identification based on rDNA ITS sequence analysis. The amplified ITS sequences of the genomic DNA (ITS1, intervening 5.8S, and ITS2) spanning around 500–600 bp were used for the identification of the fungal endophytes. All the sequences were matched against the nucleotide database using the Basic Local Alignment Search Tool (BLASTn) of the US National Centre for Biotechnology Information (NCBI), which revealed the most homologous sequences. The detailed description of the fungal endophytes with respective codes, EMBL-Bank accession numbers, and closest sequence homologs are summarized in Table 1. The identities of the endophytes were considered conspecific only at a minimum threshold identity of ≥99 % compared to the most closely related strains (Yuan et al. 2010), with the exception of only two sequences (for isolates L1 and T4) which revealed at least 98 % similarity to known reported sequences. All the endophytic fungal isolates belonged to phylum Ascomycota. Most of the isolates belonged to *Penicillium* which could, thus, be assigned as the major genus harbored in the leaves, twigs as well as buds. Other isolated endophytic fungal genus included *Chaetomium*, *Aspergillus*, and *Paecilomyces*.

Phylogeny and fungal diversity analysis

The phylogenetic tree gave a more detailed idea about the relationship between the different species of fungal endophytes obtained from different parts of *C. sativa* L. (Fig. 1). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches in the figure (bootstrap values >50 %). The tree has been drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The number of isolates obtained from different tissues of *C. sativa* ranged from 6 to 16 for twigs and buds, respectively. The species richness determined by calculating the Menhinick's index (D_{mn}) revealed that the buds were rich in endophytic fungal species (D_{mn}=1.25), followed by the leaves (D_{mn}=1.06), and finally the twigs (D_{mn}=0.81). Camargo's index depicting the tissue-specific fungal dominance was 1.23 for the twigs (highest), followed by that of leaves (0.94) and buds (0.8). The dominant species was *Penicillium copticola*, isolated from the twigs, leaves, and apical and lateral buds, with a relative proportion of P_i=0.66. The next dominant species were *Chaetomium globosum* (leaves), *Eupenicillium rubidurum* (syn. *E. meridianum*) (leaves), and *Penicillium sumatrense* (buds) with their P_i=0.06. The rest of the species were less dominant (P_i=0.03). Whole community analysis revealed that the endophytic fungal species were dispersed randomly within the host plant tissues (χ^2 *(k)* = 22.29, with *k* at 24).

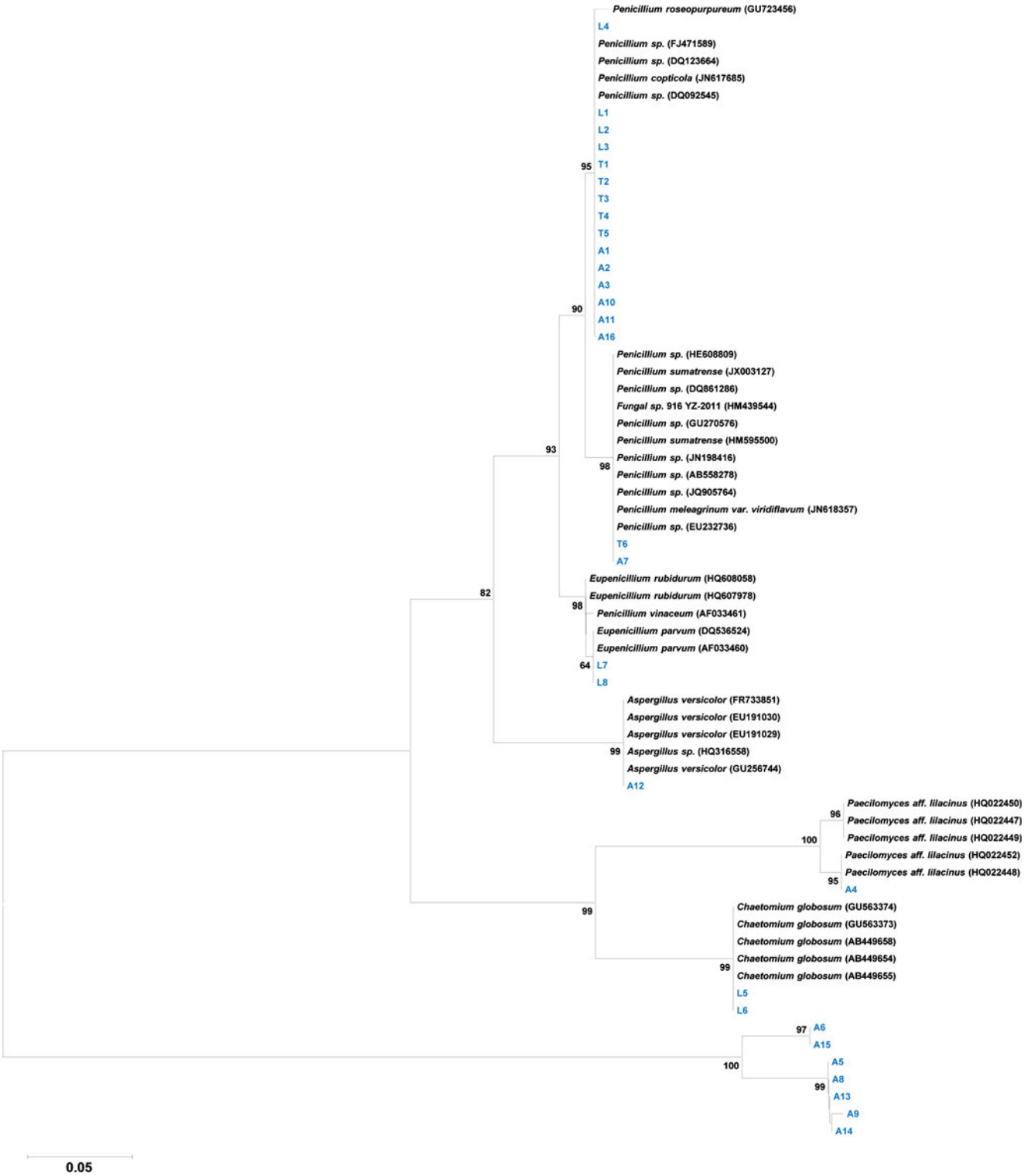

Fig. 1 Phylogenetic tree based on neighbor-joining analysis of the rDNA ITS sequences of the endophytic fungal isolates obtained from various tissues of *C. sativa*. The endophytic fungal codes are shown in *blue*. For the closely related species, the taxonomic names are followed by their respective accession numbers in brackets. Significant bootstrap values (>50 %) are indicated at the branching points. The tree has been drawn to scale

To characterize the biodiversity of our samples, we calculated Fisher's log series index (α), the Shannon diversity index (H'), Simpson's index (D) and Simpson's diversity index (1-D), and Margalef's richness (D_{mg}), respectively.

The values obtained by these tests (for leaves 1.74, 0.42, 0.28, 0.71, 7.75; for twigs 1.98, 0.34, 0.47, 0.52, 8.28; for buds 2.49, 0.45, 0.46, 0.53, 5.81) indicate that the biodiversity of fungal endophytes in *C. sativa* is not too high. The Shannon index revealed higher certainty of endophytic fungal species consistency in the twigs compared to that of the leaves and buds. Furthermore, the Simpson's index clearly showed that the leaves harbored highly diverse fungal endophytes compared to those harbored by either the twigs or the buds. Finally, Margalef's index revealed that the twigs had high taxonomic richness compared to the leaves or buds.

In vitro antagonism assay of endophytes as potential biocontrol agents

From the in vitro plate bioassay of different fungal endophytes with each of the host plant pathogen in five different types of media gave a clear idea about various types of interactions that can exist between them. Understanding endophyte-pathogen interaction is vital for understanding the biodiversity of the plant tissue microflora compared to their chemodiversity. By macroscopic evaluation of the interaction and consulting with earlier reports on various endophyte-pathogen interaction types (Miles et al. 2012; Trejo-Estrada et al. 1998), we could assign the interactions of the isolated endophytic fungi with the two *Cannabis* pathogens on five different media into 11 types (Table 2 and Fig. 2).

The percentage antagonism (growth inhibition percentage) of each fungal endophyte was calculated against each of the two phytopathogens (Chamberlain and Crawford 1999). All the growth inhibition percentages along with their respective endophyte-pathogen interaction types are summarized in Tables 3 (against *B. cinerea*) and 4 (against *T. roseum*). As expected from the OSMAC concept (Bode et al. 2002; Kusari et al. 2012b), the growth inhibition varied largely among the different fungal isolates in different media. Further, not only were diverse types of interactions between individual fungal endophyte and pathogen observed in different media, but such interactions also resulted in different degrees of growth inhibition. Almost all the endophytic isolates were capable of inhibiting, to a varying extent on different media, one or both of the host-specific pathogens with a higher extent of antagonism against

Table 2 Different types of dual culture interactions between isolated endophytic fungi and the two host pathogens (*B. cinerea* and *T. roseum*) on five different solid media

Type code	Interaction descriptions
I	Both endophyte and pathogen grow towards each other, but growth stopped as their mycelia came in physical contact; no overgrowth after mycelia contact; no inhibition zone (no halo); no color alteration of mycelia; no sporulation of endophytic fungus
II	Both endophyte and pathogen grow towards each other followed by slight overgrowth of endophyte on pathogen after their mycelia came in physical contact; no inhibition zone (no halo); no color alteration of mycelia; no sporulation of endophytic fungus
III	Both endophyte and pathogen grow towards each other, but growth stopped before their mycelia came in physical contact; no inhibition zone (no halo); no color alteration of mycelia; no sporulation of endophytic fungus
IV	Both endophyte and pathogen grow towards each other, but growth stopped before their mycelia came in physical contact and clear halo (inhibition zone) produced by the endophyte around its biomass; no halo by the pathogen; no color alteration of mycelia; no sporulation of endophytic fungus
V	Both endophyte and pathogen grow towards each other, but growth stopped before their mycelia came in physical contact and clear halo (inhibition zone) produced by the pathogen around its biomass; no halo by the endophyte; no color alteration of mycelia; no sporulation of endophytic fungus
VI	Both endophyte and pathogen grow towards each other, but growth stopped before their mycelia came in physical contact and respective clear halo (inhibition zone) produced by both the endophyte and the pathogen around their biomass; no color alteration of mycelia; no sporulation of endophytic fungus
VII	Both endophyte and pathogen grow towards each other followed by complete overgrowth of endophyte on pathogen after their mycelia came in physical contact; no color alteration of mycelia; no sporulation of endophytic fungus
VIII	Both endophyte and pathogen grow towards each other, but growth stopped before their mycelia came in physical contact and endophyte releasing visible exudates from its entire mycelial biomass; no color alteration of mycelia; no sporulation of endophytic fungus
IX	Both endophyte and pathogen grow towards each other, but growth stopped before their mycelia came in physical contact and endophyte releasing visible (colored) pigments (secondary metabolites) from the point of contact leading to complete color change of the media; no color alteration of mycelia; no sporulation of endophytic fungus
X	Both endophyte and pathogen grow towards each other, but growth stopped as their mycelia came in physical contact and endophyte sporulating profusely; no color alteration of mycelia
XI(E/P)	Both endophyte and pathogen grow towards each other, but growth stopped as their mycelia came in physical contact; color alteration of mycelia either by endophyte (E) or pathogen (P) or both (E/P); no sporulation of endophytic fungus

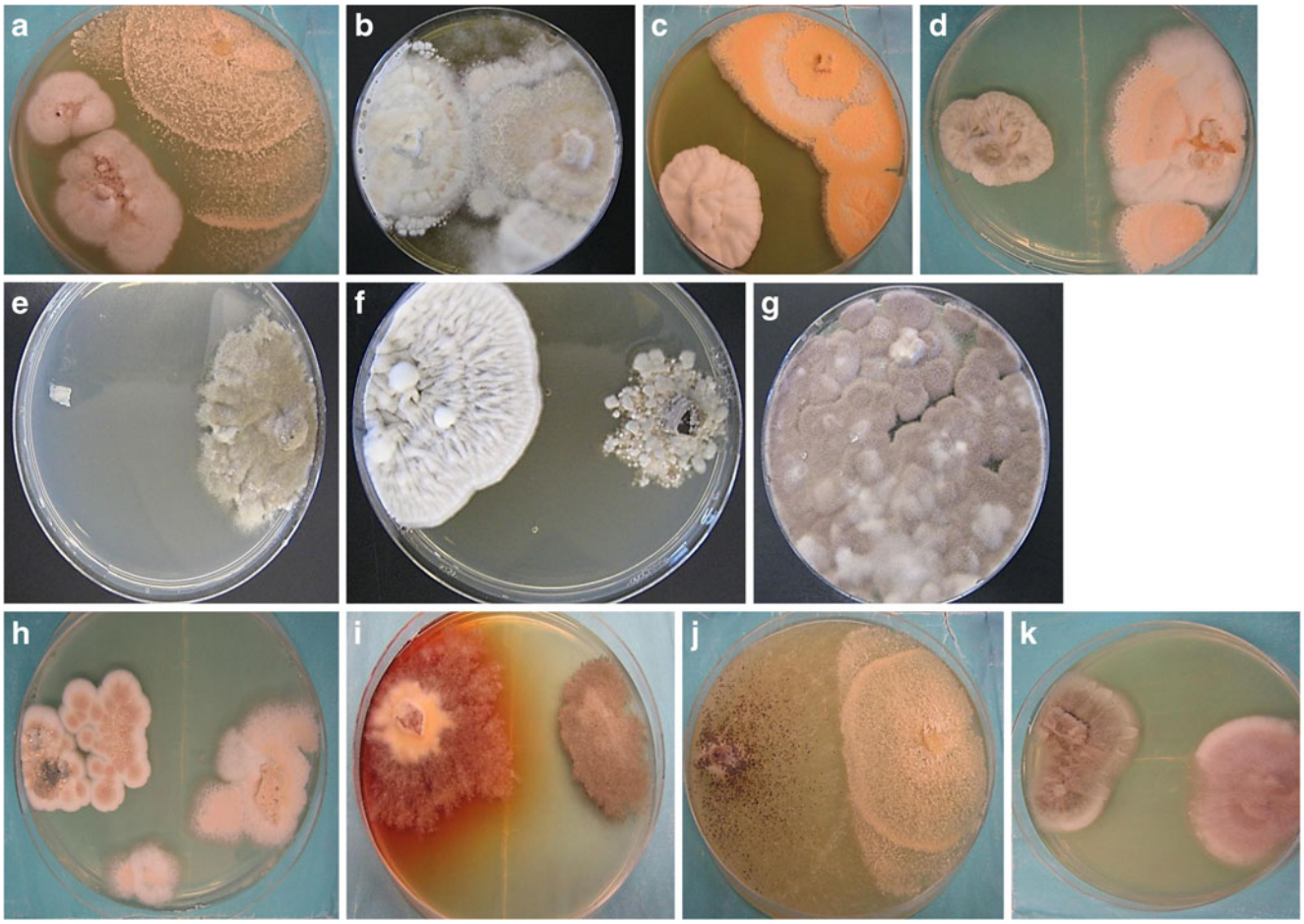

Fig. 2 Types of endophyte-host pathogen interactions observed in dual culture antagonistic assay. **a**–**k** Interaction types I–XI, where endophytes are shown on the left and challenging pathogen on the right of the representative Petri plates

T. roseum. The inhibition efficacies of the endophytes were least against *B. cinerea* on WA medium, on which mainly one type of endophyte-pathogen interaction could be observed (type III). Here, both the endophyte and pathogen grew towards each other, but their growth stopped before their mycelia came in physical contact without any visible zone of inhibition or halo, the color of mycelia remained unaltered, and no sporulation of endophytic fungus could be seen. On the same WA medium, however, the endophytes demonstrated visible antagonistic inhibition against *T. roseum*, with the endophytes isolated from the apical and lateral buds of the plant demonstrating high inhibition effects. This pattern was similar on NA medium, where the endophytes more prominently inhibited *T. roseum* than *B. cinerea*. Interestingly, most of the fungal endophytes started sporulating copiously on NA when challenged with either one of the pathogenic strains (mainly against *T. roseum*), revealing in a typical fashion the unfavorable conditions for countering the confronting pathogen. When the endophytes were challenged by the pathogenic strains on PDA and MEA media, a capricious type of interacting features could be observed that accompanied the inhibitions. The visible interaction types between the endophytes and the pathogens on SA were similar to that on WA, but the antagonistic effect on both *B. cinerea* and *T. roseum* were more pronounced. Interestingly, the endophytic fungal strain A4 (*Paecilomyces lilacinus*) could completely inhibit the growth of the phytopathogen *B. cinerea* on all tested media, and of *T. roseum* on PDA and MEA along with prominent inhibition on SA, NA and WA. The endophyte strain T6 (*Penicillium sp.*) and L3 (*Penicillium copticola*) were also dominant antagonists of the tested pathogens on one or more media.

Discussion

Over the last decades, endophytic microorganisms have garnered immense importance as valuable natural resources for imminent utilization in diverse areas such as agriculture and biotechnology (Aly et al. 2011; Rajulu et al. 2011; Kusari and Spiteller 2011; Li et al. 2012). A number of

Table 3 Growth inhibition (% antagonism) of the phytopathogen *Botrytis cinerea* by isolated fungal endophytes of *C. sativa* on five different media after 15 days, and the respective endophyte-pathogen interaction types

Endophyte strain number	Growth inhibition (% antagonism) on different media					Interaction type on different media (type code)				
	Sabouraud agar (SA)	Nutrient agar (NA)	Potato dextrose agar (PDA)	Malt extract agar (MEA)	Water agar (WA)	Sabouraud agar (SA)	Nutrient agar (NA)	Potato dextrose agar (PDA)	Malt extract agar (MEA)	Water agar (WA)
L1	33	15	50	93	20	IV	III,X	VI	III,VIII	III
L2	33	29	63	67	20	I	III,X	VI,VIII	III,VIII	III
L3	100	100	38	100	60	VII	VII,X	V,VIII	VII,VIII	III
L4	17	43	25	87	0	I,VIII	III,X	VI	I,VIII	III
L5	NI	57	0	67	NI	NA	II, X	I,IX	III,IX	NA
L6	67	57	NI	67	0	III	VII,X	I,IX	III,IX	III
L7	17	−29	25	47	0	III	III	V,VIII	III,	III
L8	−33	29	8	47	0	III	III,XI(E)	I,VIII	I	III
T1	50	15	50	32	40	III	III,X	VI	III,VIII	III
T2	20	0	50	73	20	III	III	VI	III,VIII	III
T3	30	57	55	67	−20	III	III,X	VI	III,VIII	III
T4	−3	29	88	87	0	III	III	VI	III,VIII	III
T5	−17	15	38	67	20	III	III	VI	III,VIII	III
T6	67	100	50	100	100	I,VIII	I,VII	V,VIII	VII,VIII	VII
A1	67	0	38	60	0	III	III,XI(P)	IV,VIII,XI(P)	III,XI(P)	III
A2	67	−43	48	73	0	I	I,VIII,XI(P)	VI,VIII,XI(P)	I,XI(P)	III
A3	NI	NI	NI	NI	NI	NA	NA	NA	NA	NA
A4	100	100	100	100	100	VII	VII,X	VII	VII,VIII	VII
A5	33	40	63	67	40	III	I,X	VI,VIII	IV	III
A6	50	29	50	67	0	I	I	IV	I,VIII	III
A7	17	29	63	73	−40	III	I,X,XI(P)	V,VIII	I,VIII	III
A8	33	43	25	47	0	III	III	VI,VIII	III,VIII	III
A9	17	29	38	NI	0	III	III,VIII	IV,VIII	NA	III
A10	33	29	38	67	40	III,VIII	I	IV,VIII	I,VIII	III
A11	0	43	38	67	40	III,VIII	I	VI,VIII	III,VIII	III
A12	17	15	0	47	0	III,XI(E)	I	V,VIII	I	III,X
A13	17	−20	13	53	−20	I	III,X	VI,VIII	I	III
A14	100	−57	38	67	0	VII	I	VI,VIII	III,VIII	III,X
A15	17	NI	38	80	40	I	NA	V	III	I
A16	17	15	75	87	20	I	III,XI(P)	I,VIII	III,VIII	III

NI pathogen not inhibited

NA not applicable

Negative values represent endophyte inhibited by pathogen (%)

bioprospecting strategies could be engaged in order to discover competent endophytes with desirable traits. For instance, endophytes could be isolated from randomly sampled plants from different population, or initially performing a detailed investigation of an ecosystem in order to determine its features with regard to its natural population of plant species, their relationship with the environment, soil composition, and biogeochemical cycles, followed by endophyte isolation and characterization (Debbab et al. 2012; Kusari and Spiteller 2012). Another approach could be to evaluate the evolutionary relatedness among groups of plants at a particular sampling site, correlating to species, genus, and populations, through morphological data matrices and molecular sequencing, followed by isolation of endophytes from the desired plants. Medicinal plants could also be bioprospected for endophytes, especially those plants capable of producing phytotherapeutic secondary metabolites (Aly et al. 2011; Debbab et al. 2012).

Herein we report for the first time, the isolation and incidence of endophytic fungi harbored in different tissues

Table 4 Growth inhibition (% antagonism) of the phytopathogen *Trichothecium roseum* by isolated fungal endophytes of *C. sativa* on five different media after 15 days, and the respective endophyte-pathogen interaction types

Endophyte strain number	Growth inhibition (% antagonism) on different media					Interaction type on different media (type code)				
	Sabouraud agar (SA)	Nutrient agar (NA)	Potato dextrose agar (PDA)	Malt extract agar (MEA)	Water agar (WA)	Sabouraud agar (SA)	Nutrient agar (NA)	Potato dextrose agar (PDA)	Malt extract agar (MEA)	Water agar (WA)
L1	59	47	40	65	53	III	I,X	IV	I,VIII	III
L2	36	30	27	41	53	I	I,X	IV,IX,VIII	III,VIII	III
L3	82	71	64	35	27	I	I,X	IV,VIII	I,VIII	I
L4	59	47	53	65	53	I,VIII	II,X	IV,VIII,XI(E)	I,VIII	III
L5	65	53	54	59	47	III	I,X	I,IX	II,IX	I
L6	71	47	40	53	47	III	I,X	II,IX	II.IX	I
L7	47	36	27	47	53	III	I	V,VIII	I,VIII	III
L8	47	42	53	36	40	IV	I	III	I,IX	IIV
T1	47	36	53	77	20	III	I,X	IV	III,VIII	III
T2	65	47	60	73	20	III	I	IV,VIII	I,VIII	III
T3	65	24	27	53	67	III	I,X	IV	I,VIII	III
T4	65	47	64	53	73	III	I,X	IV	I,VIII	III
T5	57	47	67	53	67	III	I,X	IV,VIII	I,VIII	III
T6	82	77	100	100	93	I,VIII	I,X	VII,VIII	VII,VIII	I
A1	59	29	49	59	71	III	I,X	IV	III.VIII	III
A2	65	41	60	82	73	I	I,X	IV,VIII	I,VIII	III
A3	NI	53	NI	NI	NI	NA	I,X	NA	NA	NA
A4	82	82	100	100	93	I	I	VII	VII	I
A5	71	52	59	53	67	III	I,X	IV,VIII	I,VIII	III
A6	77	59	67	59	40	I	I,X	III	I,VIII	III
A7	77	47	27	53	53	III	I,X	I	I,VIII	III
A8	82	53	67	53	80	III	I,X	I	I	III
A9	65	53	60	53	60	I	I	IV,VIII	I,VIII	III
A10	71	47	47	53	67	III,VIII	I	IV,VIII	I,VIII	III
A11	65	36	47	47	73	III,VIII	I	IV,VIII	I,VIII	III
A12	65	65	47	53	67	III,XI(E)	I,X	I,VIII	III	III,X
A13	65	47	67	47	60	III	II,X	IV,VIII	I	III
A14	71	47	73	71	40	I	I	IV,VIII	I	III,X
A15	77	30	40	47	67	III	II	I	I,VIII	I
A16	36	41	60	65	60	I	II,X	IV	II,VIII	III

NI pathogen not inhibited

NA not applicable

of *Cannabis sativa* L. plants. We used the bioprospecting rationale that *C. sativa* which contains a number of therapeutically relevant compounds including cannabinoids, might also harbor competent endophytes capable of providing fitness benefits to the host plant. Such benefits could encompass the endophytes producing a plethora of bioactive compounds, even the ones exclusive to the associated plant, thereby assisting in the chemical defense of the host against invading pathogens (Aly et al. 2010; Arnold et al. 2003; Debbab et al. 2012; Gunatilaka 2006; Kharwar et al. 2011; Porras-Alfaro and Bayman 2011; Rodriguez et al. 2004, 2008; Staniek et al. 2008; Strobel and Daisy 2003; Strobel et al. 2004; Suryanarayanana et al. 2009; Zhang et al. 2006). However, random screening of endophytes in axenic cultures often leads to rediscovery of known natural products, with a very high possibility of the 'cryptic' bioactive molecules not produced under normal lab conditions (Bode et al. 2002; Scherlach and Hertweck 2009). Thus, in order to screen for the most promising endophytes, we estimated the potential of the isolated endophytic fungi as biocontrol agents by challenging them with two major fungal pathogens of the host plant, *Botrytis cinerea* and *Trichothecium*

roseum. The isolated endophytic fungi were challenged by the host-specific phytopathogens on five different media, namely SA, MEA, PDA, WA and NA. The distinct types of inhibition representing the different types of antagonism (Chamberlain and Crawford 1999; Miles et al. 2012; Trejo-Estrada et al. 1998) we observed in our study revealed both the endophytic biodiversity of *C. sativa* and their potential chemodiversity in the form of producing a wide range (and/or number) of natural products with varying inhibitory activities under different media conditions. It has been well established that even slight variations in the in vitro cultivation conditions can impact the kind and range of secondary metabolites endophytes produce (Scherlach and Hertweck 2009; Kusari et al. 2012b). Recently for example, it was shown that the plant-associated *Paraphaeosphaeria quadriseptata* could start producing six new secondary metabolites when only the water used to make the media was changed from tap water to distilled water (Paranagama et al. 2007). Further, changing the medium from solid to liquid resulted in the production of radicicol instead of chaetochromin A by *Chaetomium chiversii* (Paranagama et al. 2007). Therefore, in order to verify this concept, known as OSMAC (Bode et al. 2002; Paranagama et al. 2007; Kusari et al. 2012b), we evaluated the different strategies that isolated endophytes employ against the competing pathogens on five different media. As expected, we observed a varying degree of antagonistic behavior and 11 distinct kinds of endophyte-pathogen interactions when the assays were performed on five different media. The results revealed that varying the media conditions indeed might have triggered the production of the 'cryptic' metabolites by the endophytes when challenged by the pathogens. Nevertheless, the different types and efficacies of pathogen inhibition might also be due to instability of the secondary metabolites or their reactive intermediates, a volatile nature of the compounds produced, or the compounds being produced in quantities below the minimum inhibitory concentrations (MIC) for counteracting the pathogens.

It is imperative that any plant-fungal interaction is always preceded by a physical encounter between a plant and a fungus, followed by several physical and chemical barriers that must be overcome to efficaciously establish a plant-endophyte association (Kusari et al. 2012b). It is mostly by chance encounters that particular fungi establish as endophytes for a particular ecological niche, or plant population, or plant tissue, either in a localized and/or systemic manner (Hyde and Soytong 2008). Thus, even a fungus that is pathogenic in one ecological niche can be endophytic to plant hosts in another ecosystem. It has been established for a plethora of fungi that pathogenic-endophytic lifestyles are interchangeable and are due to a number of environmental, chemical and/or molecular triggers (Eaton et al. 2011; Hyde and Soytong 2008; Schulz et al. 1999). Furthermore, groups of fungi containing large numbers of plant pathogenic species also contain large numbers of endophytic taxa. A vast majority of endophytes discovered so far are filamentous Ascomycota; this phylum comprises more than 3000 genera of mostly plant pathogens (Berbee 2001; Heckman et al. 2001; Mueller and Schmit 2007). Therefore, it is compelling that the diverse fungal isolates obtained from the tested *C. sativa* plants in the present work are selected towards coexistence with the hosts as endophytes. Interestingly for example, we found a number of *Penicillium* species exhibiting endophytic lifestyle in the associated *C. sativa* host plants (Table 1). Admittedly, only the 'cultivable' endophytic fungi could be isolated in this study and do not represent the non-culturable endophytic microorganisms of the sampled *C. sativa* plants. It should also be mentioned here that 5.8S-ITS analysis can sometimes underestimate the endophytic fungal 'species diversity' (Gazis et al. 2011), and additional parameters should be coupled to ITS rDNA sequence data before fungal isolates can be referred at the 'species' level. Further, it is highly desirable to compare the obtained ITS sequences with those from type species, when available, in order to authenticate the tentative species identification (Ko et al. 2011). Thus, the ITS-based species identification concept may not be in full agreement with the current classical concepts of *Trichocomaceae*. Nevertheless, this work can serve as the handle for further studies (both ITS-based and different other methods) on endophytes of *Cannabis* bioprospected from different other populations, different collection centers, and wild populations (when accessible) for a landscape or global scale diversity analysis.

Taken together, our results firmly revealed that the endophytic fungi harbored in different tissues of the investigated *C. sativa* plants have great promise not only as biocontrol agents against the known and emerging phytopathogens of *Cannabis* plants, but also as a sustainable resource of biologically active novel secondary metabolites. Further, it would be interesting to compare our results (which were performed using *C. sativa* L. plants from Bedrocan BV) to those of *Cannabis* plants sampled from different wild and/or agricultural populations from different parts of the world. Using the cues from the results of the present work, we have now initiated the fermentation of the endophytes in the selective media, both under axenic conditions as well as in suitably devised cocultures with the challenging pathogens, for the discovery and structural elucidation of the bioactive compounds produced by the endophytes of this plant. This would then lead us towards further mass-balance studies and gene discovery, to cross-reference the biodiversity of these endophytic fungi to their actual biochemical potential. It would, thus, be possible to completely elucidate the chemical ecology of production of target and/or non-target molecules (quantitative) by these endophytes leading to the aforementioned 'interaction types' (qualitative) with the host-specific pathogens.

Acknowledgment This work was funded by the Ministry of Innovation, Science and Research of the German Federal State North Rhine-Westphalia (NRW) and TU Dortmund by scholarship to P.K. from the CLIB-Graduate Cluster Industrial Biotechnology (CLIB^{2021}). We are grateful to the Federal Institute for Drugs and Medical Devices (Bundesinstituts für Arzneimittel und Medizinprodukte, BfArM), Bonn, Germany for granting us the necessary permissions for conducting this work (BtM number 458 49 89). We are also thankful to Bedrocan BV for kindly providing us with the *Cannabis sativa* L. plants.

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