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Daldinia bambusicola Ch4/11 an Endophytic Fungus Producing Volatile Organic Compounds Having Antimicrobial and Olio Chemical Potential

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Abstract: An endophytic *Daldinia bambusicola* isolated from stems of *Camellia caduca* (Theaceae), collected from Cherrapunji, Meghalaya, India (25.30° North, 91.70° East). Volatile organic compounds produced by this organism were analyzed through GC-MS. Major VOCs were found as linalool; benzeneethanol; 2H-1-benzopyran-2-One, 4, 7-dihydroxy; hexane; pivalic acid anhydride; *2*-ethylhexanol etc. The organism inhibited most of the test pathogens and 100 % inhibition was observed with *Phytophthora palmivora*. The organism after its treatment is able to increases the self-life of vegetables and fruits.

Key words: Antimirobial, *Daldinia*, Endophyte, Linalool, VOC.

Introduction

Volatile organic compounds (VOCs) are carbon-based solids and liquids that readily enter the gas phase by vaporizing at 0.01 kPa at a temperature of approximately 20°C [11,14]. Fungal VOCs are derived from both primary and secondary metabolic pathways [9,11]. The concentrated search for aroma producing fungi was started several years back. Many VOCs have distinctive odors so it is not surprising that interest in fungal VOCs began with the fungi that humans can smell [3,4,11]. For example, the distinct bouquets of macro fungi such as mushrooms and truffles, highly valued in the culinary arts, include mixtures of different VOCs. On the other hand, endophytes are relatively unstudied microbes that exist in plants and cause no overt symptoms or signs of their presence. Their biological diversity, especially in temperate and tropical rainforests, is enormous. Many are known to produce biologically active substances and have found application in pharmacology (e.g. the anticancer drug taxol) and agriculture [18]. In agriculture, the fungal VOCs are useful for their prospective as biocontrol agents to control plant pathogen to employ a more ecofriendly pest management strategy by reducing fungicide use on crop plants [2,7,18]. Insecticidal activity of linalool has been demonstrated by controlled-release systems for the slow volatilization of linalool [10]. In this communication we have isolated an endophytic fungus from North East India that resembled *Daldinia bambusicola*. This organism is able to produce VOCs having antimicrobial and fragrance property. The major component of VOCs was found linalool. This fungus may be useful in oleo chemical industry as a linalool producer and in agriculture as a biocontrol agent.

Materials and methods

Isolation, culture and preservation of endophyte

The endophytic strain of interest was designated Ch4/11 isolated and examined in this study were obtained from small limbs of *Camellia caduca* (Theaceae) collected from Cherrapunji,

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Meghalaya, India (25.30° North, 91.70° East). Plants were obtained by clipping terminal stem pieces and sealed in a zip lock plastic pack immediately after collection. The harvested specimens were kept cool and transported to laboratory within 72 h after collection. The specimens were kept in refrigerator at 4°C until endophyte isolation.

The stems were surface treated with sterile water and then with 70 % ethanol for 45 seconds. Stem parts were flamed to evaporate alcohol and destroy the surface microflora. Internal tissue samples of stems part were set out on plates of 2 % water agar and incubated at 25°C until endophytes become visible around the tissue samples.

Visible fungal growth from tissue samples were picked as hyphal tips and transferred to Potato Dextrose Agar (PDA) plate and incubated at 25°C. The fungus was deposited (no. 3340) at the National Fungal Culture Collection of India (NFCCI), Pune.

Scanning electron microscopy (SEM)

Isolate Ch4/11, grown on PDA plate for ten days was processed for scanning electron microscopy (SEM) in order to obtain morphological structures. For SEM sample was critical-point dried, coated with gold and images were recorded with Zeiss at10.00-20.00 kv ETH.

Qualitative analysis of volatiles

Mycelia of Ch4/11 were cultured in 250 ml Erlenmeyer flask with 50 ml sterilized potato dextrose broth (PDB), at 25°C on a rotary shaker (200 rpm) for 6 day. After separation of fungal biomass from the broth, equal amount of dichloromethane was added with it to dissolve volatile organic compounds (VOC). Mixture of VOCs with dichloromethane was separated by a separating funnel. Dichloromethane with VOCs were concentrated in a rotary evaporator. VOCs present in dichloromethane were assessed by gas chromatograph interfaced to a mass spectrophotometer. Control was prepared after adding dichloromethane in PDB without culture.

Molecular characterization and phylogenetic analysis based on ITS

Phylogenetic analysis of Ch4/11 was carried out by the approximately ~480 bp rDNA fragments and it was amplified using universal primers ITS4 and ITS5 and sequenced. Obtained DNA sequence was aligned with the available sequences of different species in gene bank by BLAST program. All desired retrieved Nucleotide sequences series were saved in a single text file in FASTA format and compiled together in a single text file. The Clustal W [19] algorithm was used to perform an initial multiple sequence alignment. Nucleotide substitution rates [8] were determined. Phylogenetic trees were constructed using the neighbour joining method [17] with CLUSTAL_X and the maximum-parsimony method [5] with the software *Fig Tree*. Alignment gaps and unidentified base positions were not taken into consideration in the calculation. The topology of the phylogenetic tree was evaluated by performing a bootstrap analysis with 100 bootstrapped trials. The phenogram obtained by maximum-parsimony analysis showed essentially the same topography as data determined by neighbour-joining (data not shown). The ITS sequence of this organism were deposited in Gene Bank, accession number KP234254.

Bioassay tests of Ch4/11 against plant pathogens

The VOCs produced by Ch4/11 were tested for their antimicrobial activity against selected pathogenic fungi [2]. A 2 cm wide strip of agar in a 100 mm PDA petri plate was removed and Ch4/11was inoculated on one side of the plate and allowed to grow 1.5-2.0 cm diameter size at 25°C. The selected fungal pathogen was then inoculated on the other side of the petri plate. The petri plate was then wrapped with parafilm and incubated at 25°C. A control plate for each pathogen was made to measure the percent of inhibition. If no growth of the test fungus was observed, test fungus was aseptically transferred on to a fresh PDA plate to determine viability.

Fumigation test of VOCs produced by Ch4/11 for fruits and vegetables storage

Culture of Ch4/11 was prepared from 10 days old colonized petri plate culture after chopping in small pieces (1 to 2 mm). Chopped culture was mixed with the sterile overnight soaked wheat. The wheat culture of Ch4/11 wear packed in

cotton bags (100 gm/ bag) and incubated at 25°C for 4 days. Fruits (banana and lemon) and vegetables (brinjal and tomato) were stored in cardboard containers (21 cm x 10 cm x 10 cm) with 4 days Ch4/11 culture containing bag for 6 days at room temperature (at about 27°C). Each fruits and vegetables wear infected by mixed fungal culture containing *Phytophthora palmivora, Geotrichum candidum*, *Sclerotinia sp, Aspergillus sp, Colletotrichum lagenarium, Rhizoctonia solani* with an injection needle. Container was observed at every 24 hours interval. Control was prepared in the same way after placing fruits and vegetables in same condition without culture bags of Ch4/11. Each experiment was done thrice.

Results and discussion

The isolated fungi in PDA plate produce whitish upright mycelium and periphery was woolly (Figure 1-A). Greenish patches appeared on the exterior after few days. The major portion of bottom side of the plate, after 10 days was greenish black with pale yellow patches (Figure 1-B). Initial growth of mycelia was rapid which lowered down after 6-7 days. Mycelia were septet and without any reproductive structure (sterile) **(**Figure 2). BLAST analysis of the 18S-ITS-5.8S region of the isolate indicated 100% sequence similarity with *Daldinia bambusicola* (Xylariaceae). The branch length of the phylogenetic tree (Figure 3) was in the same units that used to construct and infer the evolutionary distance among selected

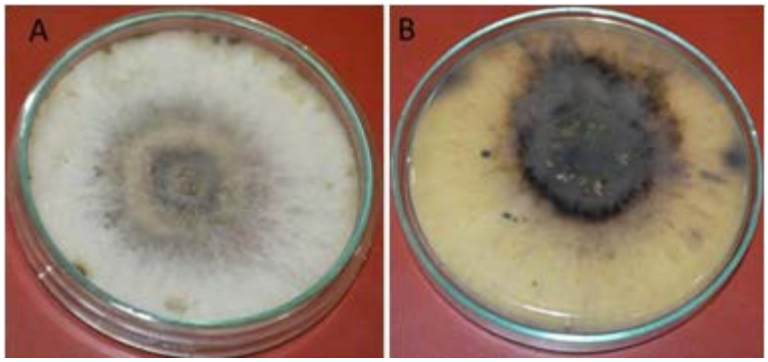

Figure 1. Plate culture of *Daldinia bambusicola* Ch4/11 in PDA media. Front (A) and back side (B) of plate

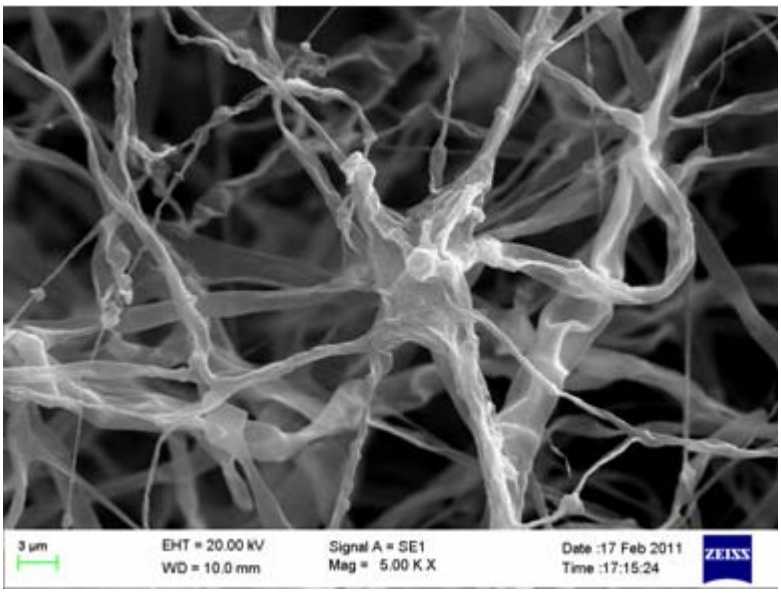

Figure 2. Scanning electron micrographs of *D. bambusicola* Ch4/11 at 5000X magnification

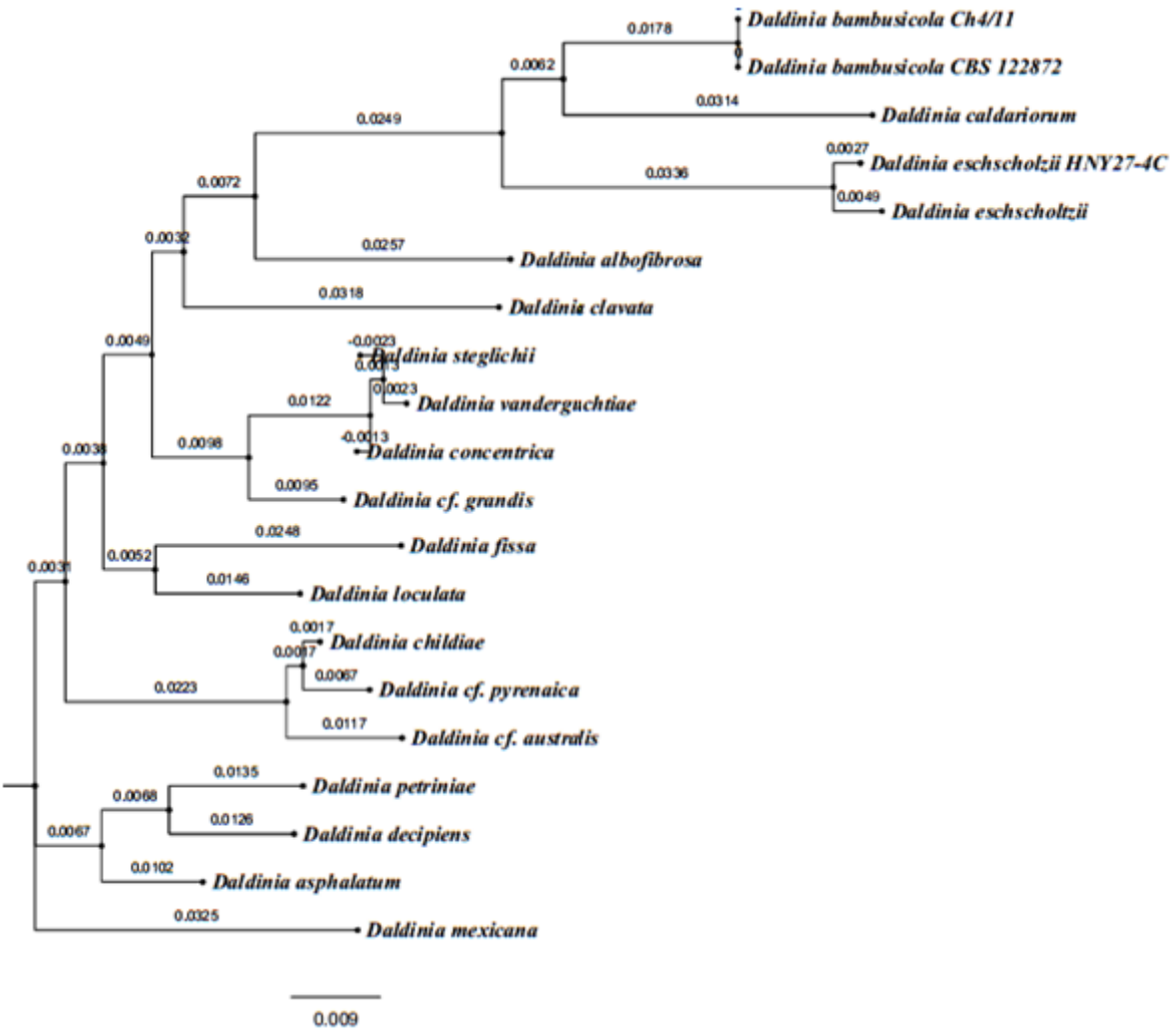

Figure 3. Phylogenetic trees were constructed using the neighbour joining and the maximum-parsimony method with the software Fig Tree to show the relationship of *D. bambusicola* Ch4/11 with selected *Daldinia* strains

Daldinia strains.

The organism Ch4/11 emits a pleasant fragrance after 4-6 days culture in petriplate. GC-MS analysis of VOCs produced by Ch4/11 showed that one component of it was a terpenic alcohol named linalool. Linalool used as an olio chemical, flavoring agent in beer, stress relief in rodents [12] and as insecticide [10]. Linalool is used as a scent in 60-80 % of perfumed hygiene products and cleaning agents including soaps, detergents, shampoos, and lotions. Linalool is also used as antimicrobial agent against pathogenic bacteria, fungi and protozoa [13,15] and it is also used in toothpaste or gargling solution [15]. Another component was linalool oxide which is a derivative of linalool, also has antimicrobial activity and olio chemical potential [21]. Esters of 4-hydroxybenzoic acid which was also present in the VOCs, reported to exhibit antimicrobial activity against bacteria and fungi [1,16] and antiviral activity [16,20]. Dominant compounds in the VOCs wear benzeneethanol and 2H-1-benzopyran-2-One,4,7-dihydroxy. The organism also produced other VOCs like hexane, pivalic acid anhydride, *2*-ethylhexanol etc. (Table 1).

Bioassay tests of *D. bambusicola VOCs* against plant pathogens

Bioassay tests were done against wide range of freshly growing plant pathogen by split- plate

Table 1. GC-MS analysis of VOCs produced by *D. bambusicola* Ch4/11after 6 days incubation at 25°C on PD broth

Retention Time(min)	Compound	Relative %	Molecular Weight (g/mol)
10.030	2-Ethylhexanol	5.815	130
13.317	Linalool oxide	0.423	170
14.197	Linalool	5.503	154
14.668	Benzeneethanol	32.448	122
16.625	Pivalic acid anhydride	4.283	186
17.949	3-hexanone, 4-Methyl	4.050	114
24.570	2H-1-Benzopyran-2-One,4,7-dihydroxy	18.638	178
25.794	Benzoic Acid,4-Hydroxy-,Ethyl Ester	5.451	166
25.511	Benzo(B)Thiophene,2-Ethyl	8.462	162
28.973	Naphthalene2,3-Dimethoxy	5.451	188
34.849	1,4-Diaza-2,5-Dioxo-3-IsobutylBicyclo (4.3.0)Nonane	9.475	210

test system (Figure 4). The basic of plant pathogen selection was to include a broad taxo-nomic representation of major plant fungal pathogen. *Phytophthora palmivora* was 100 % inhibited and died after a 4 day exposure by the VOCs of *D. bambusicola*. After a 4 day exposure to VOCs, the growth of test pathogens *Geotrichum* sp., *Sclerotinia sp, Aspergillus sp, C. lagenarium, R. solani*, *Cercospora* sp., *Alternaria* sp., *Bipolarish* sp., and *Botrytis* sp. were inhibited (Table 2). Among the test pathogens *Geotrichum* sp., *Botrytis* sp., *Alternaria* sp. and *C. lagenarium* were most sensitive to the VOCs of *D. bambusicola*, and 57 %, 60 %, 67 % and 80 % growth inhibition was recorded. However *Fusarium* sp. and *Pythium* sp. were found unaffected after exposure to *D. bambusicola*. The above out-comes showed that the VOCs of the isolated fungus were active against a widespread range of plant pathogenic fungi. The effect of same VOCs are different on different plant pathogen as there are different targets within

Figure 4. Bioassay by split-plate test method for assaying test organisms in the presence of fungal VOCs. In the represented assay, Panel A of the figure was showing bioassay of test organisms in the presence of *D. bambusicola* Ch4/11VOCs and in the panel B of the figure was showing growth of test organisms (Control) in the absence of VOCs

Table 2. Effect of VOCs produced by *D. bambusicola* Ch4/11 on various plant pathogenic fungi in a simple split plate test after 6 days at 25°C on PDA plate. Viability and inhibition of plant pathogen was measured after 48 hr exposure to the *D. bambusicola* VOCs. (+ indicates positive result and - indicates negative result)

Pathogens	Kill	Inhibit	% of Inhibition
Sclerotinia sp.	-	+	24
Geotrichum sp.	-	+	57
Phytophthora palmivora	+	+	100
Aspergillus sp.	-	+	20
Colletotrichum lagenarium	-	+	80
Botrytis cinerea	-	+	60
Pythium sp.	-	-	0
Rhizoctonia solani	-	+	14
Fusarium sp.	-	-	0
Cercospora canescens	-	+	36
Alternaria alternata	-	+	67
Bipolaris spicifera	-	+	43

the test pathogen for sensitivity [6].

Biological activity of linalool enantiomers were previously studied by Ozek *et al.*[13], and also found similar result against studied *Fusarium* sp and *Botrytis sp.* They reported that 0.3 µM linalool inhibited approximately 50 % growth of *Botrytis cinerea* at 48 hrs and no effect on *Fusarium oxysporum.* VOCs produced by *D. bambusicola* exhibited antifungal activity can be attributed for the presence of ester, naphthalene derivatives and different alcohols.

Application of VOCs Produced by Ch4/11 for fruits and vegetables storage

VOCs of *D. bambusicola* increase the storage time of fruits and vegetable and keep them fresh for more time than storage without VOCs. Inoculated mixed fungal culture was controlled by volatile compounds emitted by *D. bambusicola* (Figure 5). Exposers of VOCs protect infected tomato and banana form visible fungal growth up to five days whereas infected controlled fruits were totally rotten within three

Figure 5. Fumigation test of VOCs produced by Ch4/11 for fruits and vegetables storage at room temperature (about 27 °C) for 6 days. In Panel A and B of the figure were showing control (without VOCs) and panel C, D of the figure were showing test (With VOCs of *D. bambusicola* Ch4/11)

days. VOCs prevent fungal growth of inoculated lemon and brinjal till last day of study and remain fresh. Unexposed inoculated lemon and brinjal (Control) were decayed within four and three days respectively. VOCs of *D. bambusicola* increase the storage time of fruits and vegetable and keep them fresh for more time than storage without VOCs.

Conclusion

In this investigation it was found that *P. palmivora was* 100 % inhibited and also killed by endophytic *Daldinia bambusicola*. This strain also significantly inhibited different tested plant pathogenic fungi except *Fusarium* sp. and *Pythium* sp. VOCs of *D. bambusicola* increases storage time of fruits and vegetable and keep them fresh for more time than storage without VOCs. *D. bambusicola* Ch4/11 is a unique strain for the production of different antimicrobial compounds like linalool, linalool oxide, naphthalene derivatives, benzoic Acid, 4-Hydroxy-,Ethyl Ester etc. so it can be recommended for application in the storage of fruit, vegetables and also in soil fumigation for controlling soil born pathogens.

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